RNA silencing in GM crops: introducing a technology and assessing the risks

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Section 1 - Executive summary

A description of RNA silencing

- Ribonucleic acid (RNA) is chemically similar to the deoxyribonucleic acid (DNA) constituent of genes. Both RNA and DNA comprise a sequence of bases that carries the genetic code. Much of the RNA in a cell has a messenger role that mediates translation of the DNA genetic code into protein. However, if the RNA has an abnormal double stranded structure, it may suppress protein production or affect chromosome function through a process referred to as RNA silencing.

- The specificity of RNA silencing is influenced by the sequence of bases in the double stranded RNA: target RNAs match the double stranded RNA in 21 or more contiguous bases, although a limited number of mismatches is tolerated.

- Many aspects of the mechanisms and natural roles of RNA silencing are now well understood in animals, plants and fungi.

- In plants there are at least three variations on the basic RNA silencing mechanism. All three mechanisms are triggered by double stranded RNA that is cleaved into short silencing RNAs. One variant mechanism protects against viruses and its short silencing RNAs are known as short interfering (si)RNAs. A second variation of the mechanism silencing endogenous RNA species and its short silencing RNAs are referred to as micro (mi)RNAs. The third variant mechanism involves siRNAs but the target molecule is DNA rather than RNA. While the first two variants affect RNA after it has been transcribed, the latter impairs the transcription of RNA from the affected gene.

Potential applications of RNA silencing.

- RNA silencing biotechnology has potential utility in applications requiring specific suppression of gene expression. Results from laboratory studies indicate, for example, that a disease gene can be suppressed by introducing a double stranded copy of the disease gene RNA into a cell. Similarly the production of an enzyme can be blocked if a cell contains double stranded RNA corresponding to the gene for this enzyme.

- Applications of RNA silencing in biomedicine involve the introduction of double stranded RNA into cells. The RNA is taken up into cells and, in experimental situations, it has been used to silence viral RNAs and RNAs associated with genetic disease and cancer.

- RNA silencing can be engineered into plants using transgenes that are designed to produce double stranded RNA. In experimental situations this approach has been used to engineer disease resistance by targeting of viral RNAs. It has also been used to improve plants by silencing genes responsible for poor storage or nutritional quality of seed, fruit or tubers. RNA silencing has also been used to improve paper making quality of trees and to modify flower colour.

- It is likely that many features of crop plants can be improved by RNA silencing. The availability of the complete DNA sequence of *Arabidopsis* and rice has allowed the identification of many potential targets of RNA silencing. Silencing of these RNAs is predicted to improve yield, to increase resistance to stress or disease or to enhance the quality of crops.

Benefits associated with transgenic RNA silencing in crop plants

- A normal role of RNA silencing is to protect plants against virus infection or to regulate gene expression. Therefore the transgenic applications of RNA silencing can be considered as harnessing the natural mechanisms of genetic regulation in the crop.

- No proteins need to be expressed to achieve RNA silencing, which eliminates the hypothetical hazards associated with the presence of novel or foreign proteins in crop plants.

- Expression of viral proteins to obtain virus resistant transgenic plants is a controversial technology because of the risk of recombination events between infecting viruses and the virus-derived transgene. In contrast, short fragments of virus-derived RNA that do not contain any usable genetic information can be utilised to provide a silencing-based resistance.

- The effects of RNA silencing are genetically dominant and, as a result, they can be easily introduced into hybrid crops.

Hypothetical hazards associated with RNA silencing in crop plants

- The benefits of transgenic RNA silencing in crop plants would be offset by hazards to the environment or human health if the specificity of the mechanism is unpredictable or if the silencing effect is variable between genetically identical siblings or between generations. Environmental influences and pathogens could also affect the stability of RNA silencing.
- Hazards could also be envisioned if RNA silencing itself should prove to be prone to horizontal transfer between organisms.
- RNA silencing is frequently triggered non-intentionally in transgenic plants that were designed to accumulate a novel protein and in plants obtained by mutagenesis. The potential hazards are the same as for engineered RNA silencing.
- The potential for hazard in crop plants can be minimized or eliminated by selection of transgenic lines that are substantially equivalent to non-transgenic plants. Low hazard lines with stable silencing can be selected for empirically.
- Careful design of RNA silencing constructs will also allow potential hazards to be minimized or eliminated.

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Section 2- Introduction

More than 80 million hectares in 17 countries are currently used to grow genetically modified (GM) crops and this figure has been increasing substantially in each year since the first commercial release was approved a decade ago [1]. The continuing commercial success and an ever-growing demand for novel traits to improve quantity and quality of agricultural products on the one hand, environmental safety issues and worries about biotechnology on the consumer side on the other hand, necessitate regulation of GM crops by governments world-wide. An important part of this regulation is assessing and managing the risks involved in releasing modified organisms.

This study focuses on risks associated with a novel technology referred to as RNA interference (RNAi), post-transcriptional gene silencing (PTGS), or RNA silencing. This technology, which is described in more detail below, can be exploited to fight pathogens, control growth and development and adjust metabolic pathways. Because of the great variety of possible applications of RNA silencing, a sharp rise in the number of applications for the commercial release of silencing-based crops is expected in the near future. Furthermore, RNA silencing is frequently triggered non-intentionally in GM plants that are designed to accumulate a novel or foreign protein.

The next two sections summarise the scientific literature on mechanisms of the various RNA silencing pathways and their natural roles in plants, animals and fungi. Section 5 gives an overview of applications of RNA silencing in GM plants and a brief introduction into current developments in biomedicine. The main purpose of this report is to analyse potential hazards that might be associated with the use of RNA silencing in GM crop plants. These are presented in section 6 along with suggestions for future research.

The HTML-version of this text contains hyperlinks to a glossary and figures, which further illustrate the mechanisms involved in RNA silencing and the hypothetical hazards that are discussed in this report.

Section 3 - Summary of the literature on RNA-directed post-transcriptional gene silencing

3.1 The phenomenon

DNA makes RNA makes protein

In all living organisms, the genetic information is stored in the form of deoxyribonucleic acid (DNA) as a four �letter� code of the nucleobases adenine, thymine, guanine and cytosine (A, T, G and C). This code represents the instruction for the assembly of proteins, which perform all tasks in the cell. Ribonucleic acid (RNA) is structurally very similar to DNA, although the nucleobase thymine is replaced with uracil (U). RNA, among other tasks, serves as a messenger to transport the coded information for protein-assembly from the DNA in the cell�s nucleus to the ribosomes, the protein factories in the cytoplasm. In summary, DNA is transcribed into messenger RNA that is then translated into a sequence of amino acids to form the mature protein.

DNA makes RNA makes no protein � the silent gene

Until fifteen years ago, it was generally assumed that more copies of a gene, i.e. a DNA segment that codes for a protein, would give rise to more messenger RNA for this particular gene and therefore enhanced production of the corresponding protein. However, inserting transgenic copies of a flower pigmentation gene into the petunia genome in the early 1990s led to reduced rather than enhanced expression of the encoded protein - the flowers on these plants were either white or variegated [2,3]. It was later found out that the transgenic messenger RNA in these cases was transcribed from its DNA template but that it was degraded in the cytoplasm (Figure) before it could be translated into protein [4,5,6], hence the term post-transcriptional gene silencing (PTGS). There was also co-ordinate suppression (co-suppression) of the endogenous copies of the flower pigmentation genes.�PTGS and co-suppression are not specific to flower pigmentation genes. With viral transgenes, for example, there is co-suppression of the transgene and the viral genes and the plants are resistant to the virus [7,8,9,10,11].

We now know that PTGS represents a highly conserved mechanism in plants, animals and fungi. Various terms have been used to describe the associated silencing phenomena including RNA interference (RNAi) and quelling (in fungi). However, these terms are more historical than biologically relevant and, as the underlying mechanisms are similar, the generic term �RNA silencing� is used today. RNA silencing has become a versatile biotechnological tool in many organisms. Crop plants, for example, can be improved by specific silencing of messenger RNAs affecting growth and development, responses to stresses or the quality of the product. RNA silencing is also useful in the genetic engineering of resistance to viral and other diseases.� These potential applications of RNA silencing are described in more detail in section 5 of this report.

3.2 The basic mechanism

Double-stranded RNA triggers the silencing mechanism

In a eukaryotic cell, most of the RNA lacks the double stranded helix structure that is characteristic of DNA. Most of the RNA is single-stranded and it does not trigger silencing. However RNA silencing is triggered by RNA with double stranded regions [12,13,14]. We now know that RNA silencing associated with co-suppression and quelling is triggered by double stranded transgene RNA. One of the two strands corresponds to the sense strand of the silencing target and the other is antisense.� The silenced RNAs are either degraded or they are prevented from being translated into protein.

A messenger RNA can be specifically targeted for RNA silencing in biotechnological applications by introducing the corresponding double stranded RNA into a cell [15,16,17]. To achieve a stable long-term effect it is necessary to genetically engineer the organism to express a transgene that gives rise to a messenger RNA with features that trigger RNA silencing. The most efficient triggers are those that are transcribed into an RNA with regions that can base pair to each other to form a double-stranded structure. Such a structure is often referred to as a �panhandle� or �hairpin� and the transgenes are often referred to as RNAi constructs. Long double-stranded RNA can not be expressed in mammalian cells because it induces a strong cytotoxic reaction [18]. For this reason short-hairpin (sh)RNA constructs [19] had to be developed which are now also being tested in plants.

Another way of triggering RNA silencing in plants is by engineering a virus to carry a fragment of the target gene. Most plant viruses use single-stranded RNA to store their genetic information, which may form double-stranded structures during replication and as a result of self-complementarity between regions of the genome. As a result, the RNA silencing machinery targets the viral genome, including the inserted host gene fragment. The effect of this virus induced gene silencing [20,21] is manifested throughout the infected parts of the plant.

A small RNA is the key player

As soon as long double-stranded RNA is formed it is �diced� into small pieces of double-stranded RNA, each 21-26 nucleotides in length, named small interfering (si)RNAs. These siRNAs are the molecular hallmark of RNA silencing [22,23]. Processing long double-stranded RNA into siRNAs requires an enzyme called Dicer in animals and fungi and Dicer-like in plants [24,25,26]. There are several members of the Dicer-like protein family in plants that are each involved in RNA silencing [27,28,29,30,31,32,33].

The role of siRNAs is to guide an RNA-induced silencing complex (RISC) to RNA that has target regions with sufficient sequence similarity to the siRNA so that a stable base pairing between the two RNAs can be established (Figure). The target RNA is then cleaved within the base-paired region and RISC is free to seek another target using the same siRNA guide [34]. The target cleavage reaction is carried out by a member of the Argonaute protein family that is highly conserved in plants, fungi and animals [35].

Given the nature of siRNAs it is not surprising that introducing fragments of double-stranded RNAs as short as 23 nucleotides into plants is sufficient to trigger RNA silencing whereas fragments of 16 nucleotides or less, i.e. smaller than natural occurring siRNAs, are not [36].

In addition to Dicer/Dicer-like and Argonaute there are several other proteins involved in RNA silencing. They include double-stranded RNA binding proteins, RNA helicases that unwind the double-stranded RNA and proteins that either carry out chemical modifications of the siRNAs or that protect the siRNAs from degradation [37]. Much of the current RNA silencing research is aimed at understanding the role and mode of action of these proteins. There is also interest in understanding variations on this basic mechanism that influence the properties and outcome of the RNA silencing mechanism. In the following sections we discuss these variations.

3.3 Variations on the basic mechanism

Different mechanisms of double-stranded RNA formation

Transgenic plants may exhibit RNA silencing even if the transgene was not designed to produce double-stranded RNA. In some instances the silencing is triggered because there are at least two identical transgenes at the same integration site that are either in the same orientation (direct repeats) or in opposite orientations (inverted repeats). RNA transcripts extending across the two transgenes of an inverted repeat would have sense and antisense regions that could base pair with each other to form the double-stranded RNA trigger of silencing. [38,39,40]. Transcription of sense and antisense RNA is also possible in cases where the transgene is inserted close to a promoter of an endogenous gene that is transcribed in the opposite direction. In this case, sense RNA is transcribed from the transgene promoter and antisense from the endogenous promoter. The two can anneal to form the double-stranded trigger of RNA silencing. Similarly, silencing of an endogenous gene can be triggered by expressing the corresponding antisense strand [41].

In nematodes (roundworms), fungi and plants there is also a mechanism for producing double-stranded transgene RNA that involves an RNA-dependent RNA polymerase. This enzyme uses a single stranded RNA template to produce a double-stranded RNA trigger of silencing and, in some instances, the templates are �aberrant� RNAs lacking the structures that are present at the ends of �normal� messenger RNA [42,43]. The RNA-dependent RNA polymerase may also use �normal� RNA templates if there is a source of primary siRNAs in the cell. These primary siRNAs base pair to a �normal� RNA and prime the production of double-stranded RNA by the polymerase. The double-stranded RNA is then processed into secondary siRNAs by Dicer or Dicer-like enzymes. In this scenario a small amount of primary siRNAs leads to large amounts of secondary siRNAs. This amplification process may be important in a virus defence role of silencing.

In addition to the amplification effect, RNA-dependent RNA polymerase may also have a qualitative effect on silencing if the primary siRNAs are complementary to a localised region of the target RNA. In this scenario the priming mechanism results in secondary siRNAs that are qualitatively different from the primary siRNAs because they are complementary to the adjacent regions in the target� [44,45,46,47] (Figure). The transition from primary to secondary siRNAs has been described in plants and nematodes. However the mechanism may not be exactly the same because the transitivity is bidirectional in plants but unidirectional in the nematode. Transitivity has to be taken into account in biotechnological applications of RNA silencing because the secondary siRNAs may target messenger RNAs other than the intended targets of the primary siRNAs.

Systemic RNA silencing

In nematodes and plants the effects of RNA silencing may not be restricted to the cells in which the double-stranded RNA and siRNAs are produced. There is a systemic signal of silencing that spreads from cell to cell [13,46,48,49,50] (Figure). The nature of the signal is still unknown but since it has nucleotide sequence specificity it is believed to be RNA, probably siRNA, which might associate with specialized transport proteins [51,52]. Intriguingly, this signal moves through plants in the way plant viruses do, travelling short distances by exploiting connections between cells, the plasmodesmata, and long distances by entering the phloem, a system of �pipelines� that also distributes the products of photosynthesis throughout the plant. At the receiving end, the long-range but not the cell-to-cell signal requires the presence of an RNA-dependent RNA polymerase to start a new round of RNA silencing [53,54].

3.4 Natural roles of post-transcriptional silencing

Silencing fights viruses and viruses fight silencing

One of the major functions of RNA silencing in plants is to protect against viruses [55]. The double-stranded form of viral RNA in an infected cell is processed by Dicer or Dicer-like so that RISC recruits virus-specific siRNAs (Figure). RISC is then programmed to silence the viral RNA in these initially infected cells. In addition to this intracellular process there is also a virus-specific silencing signal that moves through the plant, either with or ahead of the virus, and impairs the establishment of systemic infection� [54,56] (Figure). To succeed in infecting the entire plant a virus must therefore suppress RNA silencing by blocking the intracellular mechanisms or the silencing signal. Consequently, viruses produce silencing suppressor proteins that interfere with the silencing mechanism [57]. The suppressors of some viruses hamper systemic signalling while others bind siRNAs, thus depleting the cell of the key component of the silencing machinery. Other suppressors may inactivate proteins involved in the silencing mechanism. Moissard and Voinnet [57] give a comprehensive overview of silencing suppressors.

MicroRNAs: small RNAs for the regulation of endogenous RNAs

Viruses and transgenes are not the only source of short silencing RNAs. In animals and plants there are partially double-stranded RNAs [58] that are processed by a Dicer or Dicer-like protein into an siRNA-like molecule, called a micro (mi)RNA [30,59,60]. This miRNA then programmes RISC so that it cleaves a target messenger RNA or blocks its translation. Each miRNA can indirectly affect many messenger RNAs because the proteins encoded by their RNA targets may be regulators of gene expression [61].� It seems that the miRNAs are an important class of regulatory RNAs acting in concert with regulatory proteins.

Plant miRNAs generally cause cleavage of the target messenger RNA, similar to siRNA-mediated silencing, whereas the normal mode of action for animal miRNAs is to inhibit translation of the target messenger RNA [34,62,63,64,65,66,67] (Figure). This difference may be because animal miRNAs are normally only partially complementary to their target sequences whereas the plant miRNAs exhibit complete or near complete match. Consistent with this idea, the mode of action of an animal miRNA - either target RNA degradation or translation suppression � can be changed by manipulating the degree of target sequence complementarity [34,68].

In one extreme example an animal miRNA was able to block translation of a messenger RNA with only 9 consecutive complementary bases [69]. This ability of miRNAs to silence partially complementary messenger RNAs has important implications for the use of RNA silencing technology in GM crops. It illustrates how transgenic or synthetic siRNAs and miRNAs may have both intended and unintended targets. This potential hazard is considered in detail in section 6.1.

Endogenous siRNAs: the dark matter of genetics

In addition to miRNAs there are other endogenous short silencing RNAs in plants. These include siRNAs that are derived from transposons [22,70]� and from repeated sequences in the genome. Transposons are mobile genetic elements with the potential to damage the genome by integrating themselves into active genes or by inducing chromosome rearrangements. It is thought that many of the endogenous siRNAs protect the genome by silencing these transposons. Trans-acting siRNAs are a second class of endogenous siRNAs. They target messenger RNAs, exactly like miRNAs, but their biosynthesis involves an RNA-dependent RNA polymerase and is similar to that of the transgene siRNAs. In a recent study it was estimated that there may be more than 75000 siRNAs in Arabidopsis of which most do not have an assigned function or target. Further analysis of these endogenous siRNAs is likely to be informative about the potential uses and difficulties associated with the biotechnological application of RNA silencing.

Section 4 - Summary of the current literature on RNA-directed DNA methylation and transcriptional gene silencing

Same trigger, different effect: silencing of transcription

Post-transcriptional RNA silencing occurs if a double-stranded RNA is similar at the nucleotide sequence level to the transcribed region of the target gene [5,9,71,72,73,74,75,76]. However, if the double-stranded RNA has sequence similarity to a promoter region that controls the expression of a gene, the silencing acts at the DNA or chromatin level and there is RNA-directed transcriptional silencing (TGS) [75,76,77].

Packing DNA more densely blocks transcription

Genomic DNA is coiled around protein-structures, consisting mainly of histone proteins. The complex of DNA and packaging proteins is referred to as chromatin. Densely packed areas, termed heterochromatin, are generally inactive, whereas less condensed regions, known as euchromatin, are more active (but there are exceptions to this rule) [78,79]. The RNA-directed transcriptional gene silencing is associated with heterochromatin formation at the targeted genomic regions (Figure).

The transition from euchromatin to heterochromatin, a process known as heterochromatinisation, involves chemical modifications of the histone proteins, such as methylation and deacetylation. The modified histones then attract proteins which condense the DNA-protein structure to heterochromatin. Such modifications can spread from a nucleation site for short distances in both directions [80,81]. In fungi (with the exception of yeast), plants and mammals heterochromatin formation is often associated with DNA methylation [82]. In insects, nematodes and yeasts there is heterochromatin but little or no DNA methylation.

DNA-methylation: a chemical modification changes gene expression

In plants, RNA mediated transcriptional gene silencing is often associated with methylation of the target promoter DNA [75] that could be either a cause or a consequence of heterochromatinisation. Methylation is a chemical modification of DNA that does not change the nucleotide sequence and is therefore referred to as an �epigenetic�, as opposed to a genetic, modification. RNA-directed DNA methylation is linked to RNA silencing by the involvement of double-stranded trigger RNA that is processed into siRNAs [83,84]. In many examples of transgene RNA silencing there is RNA-directed DNA methylation by the transgene RNA leading to methylation of a target promoter and transcriptional gene silencing.

RNA-directed DNA methylation of promoter regions is highly sequence specific and, unlike post-transcriptional RNA silencing, there is little or no transitivity: the targeted region does not extend beyond the trigger sequence [85,86]. The *de novo* DNA methyl transferases involved in initiation of RNA-directed DNA methylation in plants are the DRM methyltransferases [75,87,88,89,90]. Once initiated, the pattern of DNA methylation can be maintained, at least partially, in an RNA-independent manner by the MET1 and CMT3 DNA methyltransferases [75,87,88,89,90]. In some instances the RNA-directed DNA methylation persists through several generations [75].

The mechanism of RNA-directed transcriptional gene silencing

The best understanding of a link between heterochromatin formation and RNA silencing is in fission yeast. The heterochromatin in this organism is maintained in regions of chromosomes that influence chromosome segregation and mating type determination [91,92]. There is a clear link with RNA silencing because deletion of Dicer and Argonaute genes results in a failure to initiate and maintain the heterochromatin [93,94]. The RNA triggers of heterochromatinisation are transcripts of sense and antisense orientation that anneal to form double-stranded RNA.� This double-stranded RNA is then processed into siRNAs by Dicer [95,96]. There is also an amplification step mediated by an RNA-dependent RNA-polymerase [97]. The siRNAs are incorporated into the RNA-induced initiation of transcriptional gene silencing (RITS) complex [95,98]. RITS is like RISC in that it contains an Argonaute protein and an siRNA that guides the complex to its target. Plants and other animals also carry out RNA-directed transcriptional silencing and it is likely that they also contain RITS complexes although they have not yet been characterised.

Summary of post-transcriptional and transcriptional silencing

Post-transcriptional and transcriptional gene silencing processes are employed by plants, animals and fungi to fight viral infections, keep potentially mutagenic mobile genetic elements under control, define the chromatin status (and therefore the activity) of genomic DNA regions and to regulate temporal and spatial gene expression. In plants it is likely that there are three main pathways [99,100]:

- Post-transcriptional silencing that is mediated by siRNAs derived from long double-stranded RNA
- Pst-transcriptional silencing mediated by miRNAs, a class of endogenous small RNAs derived from specialised transcripts with short double-stranded features
- Transcriptional silencing that is associated with chromatin-remodelling

However there are probably variations on these pathways that are subdivided according to the nature of the trigger molecule and the involvement of RNA-dependent RNA polymerases. Other silencing proteins including those in the extended Argonaute family may also define variations on the three main silencing pathways.

From a biosafety point of view, specificity and stability of the silencing mechanism are of prime importance for assessing hypothetical hazards that may be associated with GM crops that carry RNA silencing constructs. These hazards are discussed in detail in section 6. The following section describes applications of RNA silencing technologies in GM organisms.

Section 5 - Applications of RNA silencing in GM organisms

5.1 Why use RNA silencing?���

In most cases, the aim of genetic engineering of crops is to improve yield, nutritional value or ornamental qualities. While some strategies require the expression of additional foreign genes, others are based on manipulating the expression of endogenous genes. Reducing the abundance of an unwanted metabolite, e.g. an allergen, can be achieved by over-producing enzymes that degrade it or by down-regulating those that produce it. Similarly, an increase in the accumulation of a metabolite can result from increased production rates or decreased degradation activity. Increasing the accumulation of an enzyme involves transformation of the plant with additional copies of the gene that encodes it, while RNA silencing can be used to specifically suppress the expression of genes.

Transgenes are prone to becoming targets of RNA silencing, which often complicates over-expression strategies while facilitating RNA silencing approaches. To express a transgene, an intact full-length copy of the coding sequence of the original gene must be obtained. In contrast, a fragment of the target gene is sufficient to trigger RNA silencing.

Although induced mutagenesis can be used instead of RNA silencing to obtain plants in which a gene no longer produces a functional protein, this approach is time and labour intensive because many plants have to be screened to find one where the gene of interest has mutated. RNA silencing, in contrast, can be designed to target the gene of interest. Furthermore, inactivation of genes by mutagenesis is permanent, while temporal and spatial control of gene inactivation is possible with RNA silencing approaches. Another advantage of RNA silencing over mutagenesis is its genetical dominance: a single copy of the transgene from one parent is sufficient to induce the silencing effect in the progeny, whereas a loss-of-function mutation has to be present in both parents to have an effect in the progeny. This greatly facilitates conventional cross-breeding with a silenced plant as one of the parents [101,102].

One very important goal of genetic engineering in crops is to increase yields by raising the level of protection against pathogens such as plant viruses. Viral diseases can not be cured in plants but insecticides are used to fight the vectors of insect-transmitted viruses and thus prevent spreading of the disease. A more environmentally friendly approach would be to enable the plants to defend themselves. RNA silencing is a natural anti-viral defence mechanism that can be used for this purpose. In nature, the silencing mechanism usually reacts to an incoming pathogen. Although this offers some degree of protection, it can not stop the disease in its early stages. Sometimes, however, parts of viral genomes seem to become integrated into plant genomes, which creates a memory for the silencing machinery that helps to target the original virus very efficiently at the early stages of the infection [103]. This is the basis for silencing-based resistance in GM crops: the plants are engineered to exhibit pre-established RNA silencing targeted at economically important viruses. This approach can probably be extended to other pathogens such as bacteria (see Table 1).

5.2 Many roads to silencing

There are several ways of triggering RNA silencing in plants. Some techniques, like virus-induced gene silencing (VIGS) or introduction of long double-stranded RNAs or siRNAs into plant tissues, elicit a short-lived silencing response that can be useful for research purposes but not for generating stable silencing in a GM crop.

Before RNA silencing was known, the most popular strategy to achieve stable silencing in plants was the �antisense� strategy. This technique involves expression of a short fragment of RNA that is complementary to the sequence of the target messenger RNA. By binding to its target, the complementary fragment prevents translation of the messenger RNA into protein and eventually causes its destruction by proteins that recognise the double stranded section [104,105].

The antisense strategy was first suggested and demonstrated in 1978 by Zamecnik and Stephenson and has been used successfully in many biological systems ever since [106,107]. In plant science, expressing antisense RNA in transgenic plants to suppress genes remained a popular technique until the dawn of RNA silencing [108]. It then became clear that antisense suppression mainly worked because double-stranded regions on the target messenger RNA trigger RNA silencing and that this could be achieved far more efficiently by directly expressing double-stranded RNA in transgenic plants [109].

5.3 Applications of RNA silencing in the literature

The first transgenic crop to be released for commercial growth made use of the antisense technology, now known to be based on RNA silencing. This was Calgene�s Flavr Svr� tomato, which was approved by the United States Food and Drug Administration in 1994 but was discontinued soon afterwards due to marketing problems and customer rejection.

In the Flavr Svr� tomato, the antisense construct was used to down-regulate polygalacturonase, an enzyme that is involved in fruit softening [110]. Flavr Svr� tomatoes can be harvested ripe and have a prolonged shelf-life. However, later examinations of the GM cultivar showed that aberrant integrations of the transgene actually triggered RNA silencing of the polygalacturonase gene by giving rise to double-stranded RNA [111].

Also in 1994, the yellow crookneck summer squash hybrid cultivar Freedom II became the first virus-resistant GM crop to be deregulated for commercial use in the United States [112]. One of its parents was the transgenic line ZW-20, which had been engineered to express the coat (RNA-packaging) proteins of two viruses: Zucchini Yellow Mosaic Virus (ZYMV) and Watermelon Mosaic Virus (WMV) [113]. Although the rationale behind the creation of line ZW-20 was to actually express the viral coat protein in order to interfere with the regulation of the viral infection (pathogen-derived resistance), the resistant plants exhibited remarkably low levels of the viral protein [114]. It is most likely therefore, that the mechanism behind the virus-resistance of ZW-20 is RNA silencing.

Although silencing-based GM crops had been introduced very early on, they do not contribute significantly to the 80 million hectares of commercially grown GM crops world-wide at present [1]. The vast majority of these crops have been engineered to express a bacterial gene conferring insect resistance or a herbicide tolerance gene.

Nevertheless, searching literature databases for applications of RNA silencing or antisense technology clearly shows that many more GM crops using these technologies are currently being developed for future commercial use. Table 1 gives an overview of the current literature on applications of silencing technologies to GM crops. Since RNA silencing is a natural defence strategy against pathogens, especially viruses, it is not surprising that a significant proportion of applications of RNA silencing in plants focus on antiviral resistance. However, the table shows that there are many other possible applications of this technology in GM crops.

It is striking that many silencing strategies are still based on antisense or sense co-suppression, i.e. silencing the target by integrating additional copies of the gene in sense or antisense orientation.� This is surprising, because it has been known for some time now that double-stranded RNA is the most potent and reliable trigger of RNA silencing. Many protocols and tools have been developed in recent years to facilitate the construction of the transgenes that are required for this strategy [115]. Double-stranded (hairpin) RNA as a trigger of RNA silencing seems to be more popular as a tool in basic research where it is used to investigate the function of genes [116,117,118,119,120,121,122,123,124,125].

At present, there are no reports of promoter-silencing strategies in GM crops in the literature. Promoters are sequences that control the expression of genes, thus silencing a promoter inactivates transcription from the gene it controls. The resulting transcriptional silencing may be advantageous because it might be more stable than post-transcriptional silencing.

RNA silencing can also be introduced non-intentionally when using random mutagenesis to obtain new crop cultivars. This was reported for a rice cultivar in which a termination signal between two genes was lost by mutagenesis. As a result, double-stranded RNA is formed, which triggers silencing of a family of genes that is involved in glutelin production [126]. This cultivar is of commercial interest because it is suitable for patients on a low-glutelin diet [127].�

�Although this report focuses on plants, the following paragraph briefly describes applications of RNA silencing in medical research.

The therapeutical usability of siRNAs is currently explored by many research groups and companies and has largely replaced antisense and ribozyme techniques [128]. The hope is that siRNAs will be routinely used one day to control metabolic, genetic and infectious diseases, by targeting over-expressed endogenes, mutated proteins, signalling proteins, proteins targeted by pathogens or pathogen-genomes themselves. Early studies in this field proved the concept but relatively large amounts of siRNAs were required which would be impractical for applications in human patients [129,130]. Hence, delivery of artificial siRNAs for in-vivo applications has been a major focus of research and a multitude of chemical modifications of the siRNA molecule have been proposed to enhance stability and cellular uptake of the drug [131]. Several studies and clinical trials are currently underway and many have delivered encouraging results in curing or relieving symptoms of conditions such as cancer, age-related macular degeneration, autoimmune diseases, arthritis and many viral infectious diseases - some of these have recently progressed to phase 1 human trials or about to do so [131,132]. The first clinical data were presented recently by SirnaTherapeutics (Boulder, CO, USA), showing promising results from the initial treatment of 14 patients of age-related macular degeneration [133].

Interestingly, we might even see overlaps between applications of RNA silencing in GM plants and therapeutical applications in human patients in the future: Zhou and co-workers reported in 2004 [134] that they had engineered tobacco plants to produce siRNAs targeting an influenza virus. They harvested RNA, including the siRNA fraction, from the plants and introduced these into isolated human cells. The plant-produced siRNAs successfully targeted the virus in the human cells and inhibited its replication.

Section 6 - Assessment of risks associated with RNA silencing in GM crops

6.1 Silencing of non-target genes (off-target effects)

The goal of silencing-based strategies in GM crops is to down-regulate a specific target gene without affecting the expression of other genes. Non-target genes can be affected by silencing trigger transgenes either directly or indirectly. A direct interaction between transgene-derived siRNAs and a non-target messenger RNA, which can occur if there is sufficient sequence similarity, can induce silencing of the non-target gene. This is also referred to as an �off-target� effect (Figure). In contrast, indirect (or �secondary�) effects on non-target genes can be caused by silencing a gene which regulates the expression of other genes. Secondary effects are a feature of any type of genetic manipulation, including induced mutagenesis. It is not always straight-forward to distinguish between primary and secondary effects because we do not know all possible interactions between genes even in organisms that have been fully sequenced.

6.1.1 Hypothetical hazards and their consequences

It is necessary to minimise off-target effects that would cause unpredictable perturbations of the plant�s metabolism. Extensive off-target effects would undermine the proposed advantage of RNA silencing as a nucleotide-sequence specific method of reducing gene expression.

6.1.2 Evidence addressing hypothetical hazards

6.1.2.1 Studies on target RNA abundance

A controversy in the current scientific literature on the issue of off-target silencing is largely based on work with animal systems investigating the specificity of miRNAs and siRNAs in RNA silencing. These studies indicate that there is a significant potential for off-target effects in RNA silencing.

The experimental approach involved analysis of messenger RNA profiles following the introduction of synthetic siRNAs into cultured cells [135,136,137]. The messenger RNAs that showed reduced accumulation in response to the siRNAs were then inspected for potential siRNA target sites. Results from studies using this approach are summarised in Table 2.� In general, affected messenger RNAs can be subdivided into those that have potential target sites with at least partial similarity to the siRNA and those that do not. A given siRNA can induce silencing of messenger RNAs that have potential target sites and we need to score these as off-target effects if they were not intended and not predicted. Those messenger RNAs that do not have a potential target site for the siRNA probably represent secondary effects, i.e. they are regulated as a consequence of silencing the intended target gene or in response to flooding the cell with the double-stranded trigger RNA. However, our knowledge of the requirements for the siRNA-target interaction may not yet be sufficient to predict all target sites. Thus, there may be messenger RNAs in the latter group that are actually directly affected by the silencing trigger but currently used computational algorithms are incapable of identifying these.

One study in human cells found that many messenger RNAs were affected by applications of synthetic siRNAs, several of these were most likely due to off-target silencing while others clearly were secondary effects [69]. As few as 10 matching nucleotides between siRNA and target were sufficient to induce silencing in at least one case. In contrast, other studies found relatively few off-target effects [138] or even none at all [139,140]. No off-target but numerous secondary effects caused by the introduction of double-stranded siRNAs was another outcome from a similar study [141]. Importantly, extensive off-target effects can be caused by very high levels of siRNAs but these are avoidable by reducing the siRNA level, a concept that has been confirmed by different approaches in plants and other organisms as well [140,142,143,144,145].

In general, the outcome of such expression profiling studies apparently depends on the experimental conditions and the choice of siRNAs and target genes. Off-target effects can not be altogether excluded but they can be minimised by optimising the experimental conditions.

An emerging theme from these studies is that the effect of a target site mismatch depends on its position within the siRNA or miRNA: Mismatches in the 5� half of the siRNA/miRNA can abolish the siRNA/miRNA-target interaction altogether while mismatches in the central and 3� positions impair the cleavage reaction. This has been confirmed in plants as well [36,142,146]. Additionally, the nature of the mismatch also influences its disruptive effect on the siRNA-target interaction: G-U wobble base-pairs and A-C mismatches, for example, are often well tolerated and G-U wobbles have even been reported to enhance the activity of siRNAs in some cases [147,148,149]. However it has not yet been possible to derive general rules for all siRNAs and their potential targets. It is likely that additional factors will need to be taken into account including the position of the target site within the target messenger RNA sequence [150,151,152,153,154,155].

So far, only a single messenger RNA profiling study in plants is available in which the issue of off-target silencing is addressed [156]. In contrast to the above studies in animal systems, Schwab and co-workers examined the specificity of miRNAs by transforming *Arabidopsis thaliana* with additional copies of four different endogenous miRNA precursor genes and the plants were shown to express elevated levels of the corresponding miRNAs. The findings differed from the siRNA studies in animals in that the down-regulated messenger RNAs all had a maximum of three mismatches to their miRNA in the target site. Similar but less extensive studies with other miRNAs produced similar results [59,157,158,159,160,161,162,163,164] and it has been suggested that the plant RNA silencing machinery may be more specific than its animal counterpart.

Even if the siRNA-target interaction would not tolerate any mismatches, off-target effects could occur because genes often share regions of highly similar sequence as a consequence of evolutionary processes by which families of genes emerge. The degree of sequence similarity varies between members of a gene family and the region within the gene. Regions that encode important catalytic domains in the final protein product are less free to acquire mutations without disrupting the function of the protein; these are therefore generally more conserved.

It is obvious from our understanding of the mechanism of RNA silencing that a prediction of potential off-target effects can only be based on a detailed analysis of the entire sequence of a potential off-target gene. However, there is a correlation between sequence similarity and the likelihood of off-target effects [165,166,167,168,169,170,171,172] and in general, as a �rule of thumb�, off-target silencing is highly likely if there is an overall sequence similarity between intended target and a gene family member of ~80% [173,174], whereas there is a low probability of off-target silencing with less than 70% similarity [173,175,176,177].

Several studies have provided a way of avoiding silencing of gene family members: messenger RNAs have regions at both ends that are not translated into protein (untranslated regions or UTRs), which usually are highly variable and thus can be targeted by a silencing trigger to ensure specificity within a gene family [178,179,180,181]. Because the target region can spread on a transgenic but not an endogenous messenger RNA [44,182], this strategy can only be used when endogenes are targeted.

6.1.2.2 �Studies on translational repression

Mismatches between siRNA and target, particularly in the centre and 3� end of the siRNA, often abolish target cleavage but that does not necessarily mean that RNA silencing is not taking place. In animals and plants, miRNAs mediate translational repression as well as target RNA cleavage. Similarly, at least in animals, there can also be translational suppression with synthetic siRNAs [68,149,183].� It is therefore possible that the analysis of messenger RNA abundance may result in underestimation of off-target RNA silencing.

To examine translational repression effects in off-target studies, protein profiling techniques are available. However, this type of analysis is far more time and labour intensive and does not offer the same extent of coverage as messenger RNA profiling.

Herman and co-workers pursued such a protein profiling approach to examine the specificity of a silencing trigger that targets a major allergen in soybean [184]. Only a small number of proteins analysed were found to be affected in the silenced plants and these were linked to the target gene. Thus, no off-target effect was found in this case.

In a different approach, introducing a mismatch in the centre of a miRNA target site in a plant in-vivo assay abolished silencing altogether instead of changing the mode of action to translational repression [142].

All in all, except for the recent evidence that at least one miRNA in plants silences its target by translational repression [159] there is not much data on this phenomenon in plants yet. It is possible that translational repression in plants is very inefficient and possibly even negligible as a potential cause of off-target effects in GM crops. Even in animal systems there is evidence that more than one target site for an siRNA is required on any given messenger RNA to induce efficient translational repression [183], which is supported by the observation that miRNA-regulated messenger RNAs in animals often contain several miRNA binding sites [62,63,67,185,186,187,188,189,190,191]. However, in at least one case in an animal system, imperfectly paired siRNA induced efficient translational repression with only a single target site [149].

6.1.3 Assessment of evidence and implications for the practice

The interaction between siRNAs and target sequences tolerates mismatches to a certain degree in animal systems. Far less is known about the details of the siRNA-target interaction in plants but the emerging evidence suggests that it is less tolerant of mismatches. Similarly, translational repression by mismatched siRNAs/miRNAs is an important issue in animal systems but less so in plants.

Several studies show that even highly inefficient mismatched triggers can induce significant silencing when introduced at high levels. To minimise off-target effects in GM crops it is thus desirable to express the silencing trigger at the lowest level possible by choosing an appropriate promoter.

To further minimise the off-target potential shorter trigger sequences should be used. Most researchers use long triggers in plants that cover large proportions of the target messenger RNA. This gives rise to a large and diverse pool of siRNAs which obviously increases the chances of off-target silencing. Constructs that produce only a single species of siRNA, known as short hairpin (sh)RNA constructs, have been developed in animal systems and these have now been adapted to plants as well [192].

Many computer programs are now available to assist in designing efficient and specific silencing triggers. A popular tool to identify sequences with sufficient sequence similarity to a given sequence is the BLAST program (see http://www.ncbi.nlm.nih.gov/Education/BLASTinfo/information3.html for details). Although this tool is frequently used by researchers to identify off-target genes, the algorithm was not designed to match short sequences like siRNAs and is therefore not ideally suited for this task [193,194,195]. To close this gap, several web-based tools are now available to analyse potential off-target effects from a given trigger sequence [196,197]. However, rice is the only crop species to be fully sequenced to date and sequencing efforts in other species are often hampered by the fact that many crop genomes are highly complex. Thus, computational prediction of off-target effects in crops will be incomplete in most cases.

The most likely candidate genes to be affected by off-target silencing are members of the same gene family as the intended target, especially if there is more than 80% sequence similarity to the silencing trigger. Therefore, it is good laboratory practice to include an analysis of the expression of known family members in any silencing experiment. Fully sequenced model species like *Arabidopsis thaliana* or rice can often help identifying gene families in less extensively sequenced species. Of course, silencing more than one member of a gene family might often be desirable to completely suppress a metabolic pathway.

While off-target effects can be minimised by applying rational design rules for the silencing trigger and making use of computational tools [193,195] it can never be completely excluded. However, unpredicted changes in gene expression patterns can be the result of other GM strategies as well, which is why the concept of �substantial equivalence� to the non-GM parent of the GM plant was introduced by the OECD and FAO/WHO to avoid exposing consumers and the environment to undesirable metabolites in GM plants [198,199]. Rapid and reliable methods are now available to prove substantial equivalence [200]. If a silencing-based GM plant can be shown to be substantially equivalent to its non-GM parent, off-target silencing can be regarded as negligible.

6.1.4 Suggestions for future research

More research is required to investigate the impact of transgenic RNA silencing strategies on whole genomes in plants. Until now only one such study has been published [156] and this involved over-expression of natural miRNAs rather than siRNAs. Natural miRNAs may have evolved for maximum specificity and it is possible that miRNA-mediated silencing is more stringent than the siRNA-mediated pathway. Therefore it is important to investigate the specificity of siRNA-mediated silencing in plants, using messenger RNA and protein expression profiling techniques to examine off-target effects caused by target cleavage and translational repression.

Such studies should also include a comparison of different trigger strategies, i.e. long or short fragments of single- or double-stranded RNA, shRNA and artificial miRNAs.�

It was reported that a shorter version of shRNAs can reduce off-target effects in animal cells [201]. This phenomenon should be examined in plants too. A comparison of silencing strategies should furthermore test a variety of promoters to find out the impact of expression levels of the silencing trigger on its off-target potential.

6.2 Silencing of target genes in non-target tissues

In some cases it might be desirable to restrict silencing of a target gene to specific tissues, e.g. if silencing of the gene in the entire plant compromises its growth and therefore the yield.

6.2.1 Hypothetical hazards and their consequences

The hazards associated with silencing of the target gene in non-target tissues depend on the function of the target gene and the reason why it was chosen to be silenced in restricted tissues only. If this was done to avoid detrimental effects on the growth and yield of the crop, silencing in non-target tissues would simply reduce the economical value of the crop while not posing any hazard to consumer or environment. If, on the other hand, the reason for the tissue restriction was that global silencing of the target gene could cause elevated levels of undesirable metabolites, silencing in non-target tissues could pose a serious hazard.

Tissue restriction is achieved by using a tissue specific promoter. This strategy could be jeopardised if the promoter has some activity in other tissues or if a systemic silencing signal can cause silencing of the target in other parts of the plant

6.2.2 Evidence addressing hypothetical hazards

The targets of silencing strategies in GM crops normally are endogenous genes. To our current knowledge endogenes are protected from the interlinked phenomena of transitive and systemic silencing [44]. This was clearly demonstrated in a grafting study where an endogenous target gene could only be silenced by a systemic signal if an additional copy, i.e. a transgene, was present in the receiving tissue [48].

Tissue specific silencing of endogenes has been used successfully in several studies [202,203,204,205]. Only in one case some leakage of silencing into neighbouring tissues was reported, which might be due to a residual activity of the promoter in these tissues [203].

6.2.3 Assessment of evidence and suggestions for the practice

Endogene silencing does not spread systemically to our current knowledge and the data from studies employing tissue specific silencing suggests that this technique produces reliable results. Tissue specificity should be carefully analysed in cases were silencing in a non-target tissue could pose a hazard. This can easily be done by standard laboratory methods.

6.2.4 Suggestions for future research

Although there is no indication that silencing of endogenes can spread systemically, only a relatively few genes have been examined so far. To verify these findings it would be desirable to test more genes using published techniques.

6.3 Stability of gene silencing

This section evaluates the likelihood that traits based on RNA silencing would be unstable or influenced by environmental factors including plant viruses.

6.3.1 Hypothetical hazards and their consequences

The hazards due to loss of RNA silencing in a GM crop depend on the function of the target gene. In many instances the loss of silencing would impair the agronomic or other properties of the crop or its product but they would not pose a threat to the environment or the consumer. However, if the silenced target is an allergen or is associated with toxicity, the instability of the RNA silencing trait could present a hazard in supposedly allergen-free food crops. Where silencing is targeted at a pathogen any unexpected loss of the disease resistance could create reservoirs for pathogens if farmers do not use any additional means of controlling the pathogen. Instability may also present a potential hazard if silencing is being used to reduce the risks associated with the expression of another transgene, e.g. by silencing the transgene in pollen to prevent any ingestion by pollen feeding animals or by using RNA silencing to confine transgenic plants to controlled environments� [206,207]. In these cases, any loss of silencing or failure to induce it would expose the environment or the consumer to a potential hazard.

6.3.2 Evidence addressing hypothetical hazards

RNA silencing may be unstable because it fails to be initiated in the plant. It could also be inactivated because expression of the silencer transgene exhibits spontaneous instability manifested within a plant or over several generations.� Furthermore, there might be environmental factors including stress or virus infections that influence transgene RNA silencing.�

6.3.2.1 Variability of the onset and extent of silencing

Many examples of RNA silencing involve transgenes that are a copy of the target gene in either a sense (co-suppression) or antisense orientation (antisense suppression).� Embryonic and meristematic tissues at the growth tips are often free of silencing in plants with these constructs [208] and the onset of silencing occurs spontaneously and unpredictably at various stages of the plant�s life cycle [2,3,173,176,177,209,210,211,212,213,214]. In some of these cases the silencing was shown to be manifested only in a subset of the plants carrying the same transgene locus (Figure).

The onset and patterning of silencing in plants with sense and antisense transgenes may be influenced by the expression level of the transgene or its target in different tissues of the plant. The Cauliflower mosaic virus (CaMV) 35S promoter is the most frequently used element to control the expression of transgenes, including silencer transgenes. Although CaMV35S generally is a strong promoter in most tissues of dicotyledonous plants, some variation in its activity, particularly between roots and leaves, has often been observed [215,216,217,218,219] and could account for the difference in silencing-based virus resistance between aerial parts and roots of a plant [220]. There may also be seasonal influences in 35S-driven transgene expression in perennial plants [221], which might influence the efficacy of a silencer transgene.

The pattern of silencing in plants with sense transgenes could also be influenced by the spreading of a systemic silencing signal. In plants exhibiting co-suppression of nitrate reductase the silencing causes a yellow chlorotic effect so that the spread of silencing could be monitored by the spread of chlorosis [211]. Silencing was initiated in small regions of young leaves and it later spread through the phloem following the flow of photo-assimilate from source leaves to sinks. From these findings it seems likely that the timing and pattern of silencing would vary depending on factors affecting phloem transport.

In contrast to weak triggers of silencing like sense or antisense single-stranded RNA, double-stranded RNA is a highly potent trigger and plants that express double-stranded silencing triggers usually exhibit fully established silencing in all young seedlings of a transgenic plant line [115,121,222].

Once established, RNA-silencing can be lost again if the silencer transgene undergoes transcriptional inactivation (Figure). Transgenes have indeed often been shown to be unstable due to becoming hypermethylated in a process that can take several generations� [223,224,225,226,227] (Figure). This process could be more efficient in cases where the transgene is specifically designed to trigger silencing, thus also promoting its own hypermethylation.

Several studies have shown that silencer transgenes trigger RNA-directed DNA methylation against themselves [43,228,229,230].� Although methylation of transgenic DNA can spread from its original target region, it does not normally cross the border between transcribed regions and flanking sequences such as promoters [231,232,233,234]. However, the presence of several copies of the original methylation target site in the genome can cause more extensive spreading of methylation into neighbouring genomic regions [235,236]. While hypermethylation in the transcribed region of the silencer transgene might even increase its silencing efficiency, spreading of the methylation into the promoter (Figure) would inactivate the silencer and thus re-activate the silenced target gene.

To our knowledge, there are no reports in the literature that clearly show a loss of silencing due to promoter hypermethylation during the lifetime of a plant. Using co-suppression strategies, silenced leaves have been reported to occur between non-silenced ones and vice versa [177] and one study even found an entire axillary shoot on an otherwise completely silenced plant that seemed to have lost silencing [237] but it is unlikely that silencing was lost as a consequence of transcriptional inactivation of the silencer transgene because the latter was found to be over-expressed. It seems more plausible that silencing had failed to initiate in these tissues. The same is true for the variegated silencing phenotype of flower pigments in petunia [2,3,5].

6.3.2.2 Stability of RNA silencing over generations

Post-transcriptional silencing appears to be stable during the life-time of a plant but, in contrast to transcriptional silencing, it is lost in reproductive tissues or during seed development and re-established in the progeny with the same frequency and spatial/temporal pattern as in the parental generation [176,214,238,239,240] (Figure). As discussed above, silencer transgenes may become inactivated in a gradual process involving increasing levels of methylation over the course of several generations The same effect has been observed as a consequence of vegetative propagation [241].

Several authors reported that transgene-induced silencing was reliably re-initiated in the progeny and stable during the life time of a plant for at least 3-5 generations [116,117,118,119,120,121,122,123,202,205,208]. However, there are also reports where silencing failed to re-initiate after seed- or vegetative propagation or showed increased variability [124,125,241,242] . It is likely that the loss of silencing was caused by a switch from post-transcriptional to transcriptional silencing of the silencer transgene in these cases.

There are not many long-term observations of silencing stability but some plant lines with co-suppression phenotypes are used by many research groups and must have been seed-propagated many times since they were generated. One particularly well examined example is the tobacco line 271 which harbours a silencer transgene that triggers post-transcriptional silencing of endogenous nitrate reductase genes and transcriptional silencing of viral promoters. This line was obtained in 1992 and is still in use in various research groups, thus silencing has been stable in this line for an unknown number of generations over the last 13 years [243,244].

Another long-term observation comes from a rice cultivar with an inverted-repeat re-arrangement within its genome which has shown stable silencing for more than 20 generations now [126]. In addition, there is evidence that naturally occurring integration of viral genes into a plant genome can �immunise� the plant by providing a memory for a silencing-based resistance. This phenomenon appears to have been stable in a *Nicotiana* species for an extremely long period of time, that was sufficient to cause extinction of the original virus [103].

Silencing-based GM crops can be useful in classical cross-breeding programs. Several studies examined the silencing phenotype in plant lines into which two different silencer transgenes had been introgressed and found that these �stacked� transgenes gave rise to the same pattern of silencing as in the parental lines they were derived from� [176,245,246]. In one case, highly variable silencing patterns were reported in a plant obtained from crossing two antisense-suppression lines [247]. However, these were probably caused by growing the plants in in-vitro culture and not by the cross-breeding procedure itself.

6.3.2.3 Viral infections

When two unrelated viruses infect a plant, one of the two is often found to accumulate to higher than normal levels, a phenomenon known as �synergism� [248]. It has been shown that the virus showing increased abundance benefits from the silencing suppressor encoded by the other virus [249,250,251,252]. Thus, viruses can �co-operate� to overcome the natural resistance based on RNA silencing. In addition, viruses have also been shown to interfere with some forms of natural occurring co-suppression affecting flower or seed pigmentation [253,254,255]. Consequently, it is likely that virus infections can result in a loss of transgene-induced RNA silencing in a GM crop (Figure).

Such loss of transgene-induced silencing or prevention of its initiation has indeed been demonstrated in several experimental systems in plants and this has been developed into a tool to identify and characterise novel silencing suppressor proteins� [22,56,251,252,256].� A virus may not easily overcome a silencing-based resistance by suppressing the silencing because it s targeted for degradation by the silencing machinery before it can accumulate sufficient amounts of its suppressor protein. However, it has been shown that a previous infection with a non-target virus can suppress the silencing against the target virus and allow the latter to infect the plant [257,258,259,260].

Another way in which a virus can inactivate silencing is by transcriptionally silencing the promoter that drives the expression of the transgene that triggers silencing. This requires an infection with the virus from which the promoter was derived, the Cauliflower mosaic virus in most cases, or a very closely related virus. Transcriptional silencing of transgenes under the control of the Cauliflower mosaic virus 35S promoter as a consequence of an infection with this virus have indeed been found [261].

Viruses can also influence the silencing machinery by inducing a stress response in the plant as discussed below.

6.3.2.4 Stress and other environmental influences

Stress can be induced by sub-optimal environmental (abiotic stress) parameters such as temperature, light and chemical composition of the soil or it is caused by other organisms (biotic stress) that cause injuries or disease. Stress induces widespread changes in the plant metabolism, which also affect the RNA silencing machinery.

A viral infection, in addition to actively suppressing silencing in many cases, triggers stress responses similar to those triggered by other forms of biotic or abiotic stress [262,263,264]. In one report, a virus escaped RNA silencing when the plants were infected with various unrelated viruses [265]. This loss of virus-resistance did not depend on a silencing suppressor and was also induced by abiotic stress.

Another inducer of stress responses is growth on artificial media, a technique that is often used to vegetatively propagate plants. Callus culture is the induction of a tumour like growth from pieces of plant tissue on artificial media which can be used to transform and regenerate plants. Loss of silencing or reduced silencing efficacy, even when using highly potent triggers, has been reported several times as a result of callus culture or in-vitro propagation [222,241,266].

Callus- and in-vitro culture, similar to other stress conditions, induce increased global methylation rates of genomic DNA [241,267,268], which could facilitate the self-inactivation of silencer transgenes due to promoter methylation and thus re-activate the silenced target gene.

However, the effect of callus and in-vitro culture can not be generalised as there are reports that show that a callus was not only able to stably maintain silencing but also to initiate silencing of another target [269]. Furthermore, in-vitro culture was even used in some cases to increase the efficiency of silencing initiation [211,213].

Changes in environmental conditions influence the metabolism of plants even if they are not extreme enough to cause a stress response. One environmental parameter that is now well known to influence the efficacy of RNA silencing is the temperature. Higher temperatures generally lead to increased abundance of virus- and transgene-derived siRNAs and thus enhanced silencing efficacy, which can be exploited to cure plants of viral infections [270]. In contrast, low temperatures inhibit the activity of the silencing machinery [271,272,273], although miRNA biosynthesis is unaffected [271]. In addition, transcriptional silencing by hypermethylation is less efficient at low temperatures [274].

6.3.2.5 Evidence obtained from field trials

Field trials are more relevant to the agronomical use of GM plants than studies in the laboratory because the expression of transgenes can be greatly affected by environmental factors [275,276]. In addition, large numbers of transgenic plants can be grown and analysed in field trials and these are exposed to pathogens and environmental changes. Therefore, a field trial summarises all of the above issues in one experiment and allows statistical analyses.

No reports of field trials with plants expressing highly potent double-stranded RNA silencer transgenes seem to be available yet but some trials are ongoing at the moment or planned for the near future (see: http://www.ogtr.gov.au/rtf/ir/dir054secv.rtf and http://gmoinfo.jrc.it/gmp_report_onepag.asp).

All of the studies discussed here used plants in which silencing was triggered using either the co-suppression or antisense strategy. Field trials confirmed laboratory results that the onset of silencing with these weaker silencer transgenes can be variable and often occurs at late stages of plant development [168,170,211,213,214,276]. In one study, there was increased variability in the level of co-suppression of flower pigment genes in comparison to laboratory experiments [276]. Furthermore, the onset of silencing was shown to be affected dramatically by the growth conditions [211,213,214]. None of these studies analysed individual plants for spontaneous loss of silencing but the percentage of silenced plants never decreased in any field trial over time, thus if there was any loss of silencing it did not affect significant proportions of the plants analysed [170,213,214]. Additional indication for the long-term stability of engineered silencing phenotypes comes from studies describing silencing-based pathogen resistance, many of them under field conditions [113,114,277,278,279,280]. However, these experiments are not as informative as the above studies because the silencing phenotype, unless examined in the laboratory, is only apparent when the plant is under pathogen attack.

Field trials with transgenic trees are an opportunity to monitor the stability of silencing in individual plants over particularly long periods of time. In two studies, silencing was found to be stable over a period of four years when the trials were terminated [277,281].

6.3.3 Assessment of evidence and implications for the practice

It is clear from the above that the choice of a silencing trigger greatly influences the various aspects of RNA silencing stability. Surprisingly, many researchers still use the rather inefficient co-suppression or antisense-suppression strategies although a number of tools and protocols is available now to simplify the construction of efficient double-stranded RNA silencer transgenes [115]. Using these highly potent triggers generally results in more stable and reliable silencing than co-suppression or antisense-suppression. Consequently, double-stranded RNA triggers should be used in any case where stable and reliable silencing throughout the plant is critical for biosafety reasons. In these cases, the silencing phenotype should also be analysed in different tissues of the plant as there may be significant variations in the silencing efficacy, particularly between roots and aerial parts.

Spontaneous loss of silencing during the life time of a plant has not been ruled out yet but in most cases it is more likely that silencing that seemed to have been lost had instead failed to initiate due to an ineffective silencer transgene.

To ensure long-term stability of a silencer transgene, it is important to prevent its promoter from becoming hypermethylated and thus inactive. Strategies have been developed to increase the stability of transgenes in GM plants which might be even more crucial for silencer transgenes. These include avoiding excessive bacterial vector sequences flanking the transgene in the delivery vector and selection for single integration sites within gene-rich genomic neighbourhoods with low levels of methylation and heterochromatin [282,283].� Transgenes can be embedded in sequences that target a region to transcriptionally active sites within the nucleus [284]. These so-called 'matrix attachment regions' shield the transgene from the influence of neighbouring heterochromatin. A recent study clearly showed that post-transcriptional silencing is significantly more stable over the cause of several generations when matrix-attachment regions are included in the construction of the silencing trigger transgene [279].

Due to its inheritability even in absence of the original trigger, transcriptional silencing might seem to be preferable to post-transcriptional silencing in terms of long-term stability [75]. However, only a subset of the progeny actually inherits the silenced state [75,230,285,286]. Therefore, transcriptional silencing does not increase the long-term stability of the silenced phenotype in comparison to post-transcriptional strategies.

Even stable silencing that is induced by strong triggers can be impaired or lost in response to environmental conditions or pathogen attacks. Viral infections are the most serious threat to the long-term stability of RNA silencing because viruses often encode silencing suppressor proteins. In cases where the target of a silencing strategy is a virus, this effect would require a double infection because the target virus itself is unable to establish an infection and express its silencing suppressor. The likelihood of such double-infections depends on the crop and regional conditions.

In summary, most reports so far indicate that silencing is stable under field conditions but there is no way of excluding unpredicted fluctuations in the efficacy of RNA silencing or even a complete loss of silencing under certain conditions.� In most cases this would not pose a hazard to the environment or the consumer because a loss of silencing simply re-activates the production of a natural metabolite. However, to our current knowledge, RNA silencing can not be recommended for any applications where any instability of silencing would cause serious hazards to the consumer or the environment.

6.3.4 Suggestions for future research

Most of the studies reviewed in this section involved co-suppression or antisense suppression strategies. Double-stranded RNA triggers, on the other hand, have shown far superior properties in all studies so far but more data is required to assess their long-term stability. These studies must take into account the different integration sites and the structure and length of the trigger sequence. As discussed in section 6.1 shorter triggers are preferable to avoid off-target effects. Although shRNA triggers have recently been adapted to plants [192], no study on their long-term stability in plant genomes is available yet.

Furthermore, field trials with plant expressing highly potent silencer transgenes are required to further assess their stability. A useful model system would involve silencing of a gene that has an easy-to-score phenotype but is dispensable for normal plant development, e.g. genes involved in flower pigmentation.

6.4 Escape of viruses from silencing-based resistance

Being targeted by RNA silencing in a GM crop imposes a strong selection pressure on the virus to reduce the similarity between its genome and the silencing trigger by acquiring mutations. It is highly likely that viruses are able to escape from being silenced in this way because of the high mutation rates associated with viral replication, especially in RNA viruses (Figure).

6.4.1 Hypothetical hazards and their consequences

Obviously, loosing its resistance due to viruses evading silencing by acquiring mutations would seriously impair the agronomical benefit of the GM crop but not necessarily pose a hazard to the environment or the consumer. However, forcing a virus to acquire mutations can facilitate the emergence of novel viruses which might be a threat to other crops. A breakdown of resistance in a supposedly resistant crop could also create a reservoir for the virus that might pose a threat to plants on nearby fields.

6.4.2 Evidence addressing hypothetical hazards

An escape of viruses targeted by transgene-induced RNA-silencing has not been reported yet in plants. However, viruses were shown to escape from being silenced by very short triggers of silencing (siRNAs in this case) in mammalian cells. The escaped viruses had indeed acquired mutations in the targeted regions [153,287]. However, it was also shown that this effect could be avoided by targeting several regions at once [153].

6.4.3 Assessment of evidence and implications for the practice

It is likely that viruses can escape from being targeted by RNA silencing in plants if short triggers are used. Most silencing strategies in plants employ long triggers that target large regions within the viral genome. However, as discussed in section 6.1, short triggers are preferable to reduce off-target effects. A compromise would be to target several regions within the viral genome with short triggers such as shRNAs as shown for mammalian systems.

6.4.4 Suggestions for future research

Experiments similar to those done in mammalian systems [153,287] need to be carried out in plants to assess the risk of viruses acquiring mutation to escape from a silencing-based resistance in GM crops.

6.5 Saturation of the silencing machinery

Transgenic RNA silencing strategies flood the organism with silencing trigger molecules to induce efficient suppression of the target gene. This basically mimics a viral infection during which siRNA levels reach exceptionally high levels [288]. The consequence could be overloading of the silencing machinery, which may be tolerated temporarily but might result in long-term defects (Figure).

6.5.1 Hypothetical hazards and their consequences

Saturating all available silencing effector complexes by an overload with transgene-derived siRNAs could render the plant more susceptible to virus infections and cause developmental defects due to an interference with endogenous small RNA functions. Since one of the major functions of endogenous siRNAs is to keep transposable elements under control, this could lead to enhanced rates of mutation [289,290,291,292].

6.5.2 Evidence addressing hypothetical hazards

No direct evidence for saturation effects in plants has been reported yet. In one study such effects where analysed in plants that expressed two different double-stranded trigger RNAs. There was efficient silencing of both endogenous targets and thus no saturation due to stacking of the two trigger transgenes in this case [204].

In animal systems, however, there is clear indication for saturation effects. Silencing two target genes simultaneously in the roundworm *C. elegans* dramatically reduces the efficiency of silencing compared to silencing of one target alone [293]. Similarly, administering synthetic siRNAs targeting two different RNA viruses in mice was shown to successfully inhibit both. However, excessive amounts of one of the siRNAs compromised the effect of the other one [294]. It has furthermore been demonstrated in vitro and in vivo that the silencing effector complex (RISC) can be saturated [295,296].

6.5.3 Assessment of evidence and implications for the practice

It is not surprising that the silencing machinery can not cope with indefinite amounts of trigger and mediator molecules, so saturation effects have to be expected. These will depend largely on the individual construct and transformation event. One consequence of a saturated silencing machinery would be a loss of natural silencing-based resistance to viruses. Thus, a simple test would be to infect the GM plant with an array of viruses that replicate in that species and are known to be targeted by RNA silencing. Comparing the overall susceptibility of the plant and the abundance of virus-derived siRNAs with a non-transgenic control gives an indication as to wether or not the silencing machinery is saturated in the transgenic plant line.

Very low levels of siRNAs, which are not sufficient to trigger post-transcriptional gene silencing, might be sufficient to efficiently induce methylation and therefore transcriptional silencing [286]. Thus, targeting promoters for transcriptional silencing might be an alternative strategy to avoid saturation effects if necessary.

Most consequences of saturation effects would affect the economical value of the GM crop while not posing any hazards to the consumer or the environment. An increased mutation rate, however, would lead to unpredictable long term changes to the GM crop which would be undesirable. Defects in miRNA-mediated gene regulation due to saturation could also have an impact on metabolic pathways that are normally regulated by miRNAs, thus changing the metabolite composition of the plant.

6.5.4 Suggestions for future research

Saturation effects have not been analysed in detail in plants yet. It is particularly important to investigate the possibility of an increased mutation rate as outlined above. The effect of constant high levels of transgene-derived siRNAs on endogenous small RNAs and their efficacy should also be analysed.

6.6 Horizontal transfer of silencing

Horizontal gene transfer is a major concern with GM crops. Silencing-trigger constructs are not different from any other type of transgenes. Horizontal gene transfer in general has been reviewed extensively elsewhere and is beyond the scope of this report.

One related issue, however, is peculiar to silencing based GM plants: because the silencing machinery is so highly conserved between species, any silencing trigger or siRNA might therefore induce silencing in a non-target organism if it is transferred between organisms. We refer to this as horizontal transfer of silencing (Figure).

6.6.1 Hypothetical hazards and their consequences

Horizontal transfer of silencing could induce unpredictable effects in non-target organisms, which would seriously undermine the biosafety of silencing-based GM crops.

6.6.2 Evidence addressing hypothetical hazards

There is no difference in the structure of siRNAs generated in different organisms. Consequently it is possible to induce specific silencing in human cells using siRNAs that were produced in a transgenic plant [134]. However, it is necessary to use a concentrated RNA extract from the plant, which is then applied to isolated human cells in a cell culture environment to induce silencing. Therefore, no hazard to the consumer can be implied from this study.

A major concern with horizontal gene transfer is the exchange of genetic information between GM plants and bacteria. However, despite having regulatory small RNAs and some of the protein components that function in RNA silencing in higher organisms, bacteria do not employ the same RNA silencing mechanism as higher organisms [297,298,299]. Therefore, horizontal transfer of RNA silencing from plants to bacteria is highly unlikely, albeit not formally ruled out by experiments yet.

Nematodes (roundworms) are more likely candidates for a horizontal transfer of silencing from plants to other organisms because they are very abundant in soil and many feed on plants. In the nematode *C. elegans*, silencing can be triggered simply by feeding the animals on bacteria that express double-stranded RNA [15,143,293]. Ingestion of the double-stranded RNA induces silencing in the worm and its progeny. Similar to the experiment described above, plant-generated siRNAs can also be used to trigger silencing in *C. elegans* [300]. These� experiments have not been repeated yet with plant feeding nematodes but it was demonstrated recently that silencing can be induced in a root-knot nematode [301] and it has been suggested that this could be exploited to develop a novel type of silencing-based nematode resistance in plants [302] .

A transfer between plants is also possible in an experimental system: in one study, silencing was triggered in a plant by infiltrating leaves with an extract containing siRNAs from a silenced plant [303]. Rubbing extracts from bacteria that express a silencing trigger onto plant leaves also induces silencing [304,305].

6.6.3 Assessment of evidence and implications for the practice

Although it has been shown that siRNAs and triggers of silencing can be transferred from one organism to another, we have no indication that this actually occurs in nature. Although plant-generated siRNAs have been used to trigger silencing in human cells, a horizontal transfer of silencing from silencing-based GM crops to humans or animals is unlikely because a high dosage of extracted plant RNA was required in the experimental system to trigger silencing. Furthermore, siRNAs that are not chemically modified are unstable in the blood stream, which has been a major obstacle for the development of siRNAs as therapeutics. For this reason, any plant-derived siRNA that would survive a gut passage would quickly be degraded in the blood. Even if some siRNAs would be taken up by cells, any silencing they might trigger would be short-lived because RNA silencing in humans does not involve an amplification step.

A more realistic scenario is the transfer of silencing from plants to silencing-competent soil organisms such as nematodes. However, there is no experimental indication yet that this transfer is possible.

A transfer of silencing between plants would also be undesirable. The techniques described in the literature to achieve such a transfer involved extracts containing large amounts of silencing triggers or siRNAs. It is unlikely, although not ruled out yet by experiments, that a mechanical transfer of silencing from plant to plant is possible. In addition, there seems to be no systemic silencing and amplification of silencing when endogenes are targeted. Any silencing of an endogene by a horizontally transferred silencing trigger or siRNA would therefore be weak and very short-lived.

Horizontal transfer of silencing, even if it should be possible in nature, would only have an effect if there are suitable target genes in the receiving organism. Thus, measures that reduce the likelihood of off-target effects in the target organism, such as using short triggers, could also reduce the likelihood of effects in a non-target organism if transfer would indeed occur.

6.6.4 Suggestions for future research

So far, a horizontal transfer of silencing from a GM crop to other organisms has not been observed in nature. Initially, the most likely target organisms have to be identified and assays have to be developed. Induction of silencing has recently been achieved with a root-knot nematode. Plants that express a silencing trigger directed at a nematode gene can be used to test the transfer of silencing between the two organisms.

Phloem-sap sucking insects might also transfer silencing signals between plants. An experimental system needs to be established to test this possibility.

6.7 Risks associated with non-intentional RNA silencing

Non-intentional RNA silencing is frequently associated with transgenic over-expression strategies in plants. In some cases this may be obvious because the transgene is not expressed but there may also be a background level of RNA silencing even though the transgene is expressed. This may easily be overlooked in cases where RNA silencing was not part of the design.

6.7.1 Hypothetical hazards and their consequences

Non-intentional RNA silencing in plants that were designed to express a transgene is mechanistically identical to intentional silencing using trigger constructs. Therefore, the hypothetical hazards and their consequences are basically the same as those identified above for GM crops in which RNA silencing is triggered intentionally. The main concerns in this case are �off-target� effects on endogenous genes, saturation of the silencing machinery and a hypothetical transfer of silencing between organisms.

6.7.2 Evidence addressing hypothetical hazards

All of the evidence addressing hypothetical hazards from intentional RNA silencing discussed above applies to non-intentional RNA silencing as well. However, it is important to investigate the likelihood and magnitude of non-intentional RNA-silencing in GM crops. It has been known for a long time that complete or partial silencing of the transgene is a frequent outcome of plant transformation even if the transgene is not designed to give rise to double-stranded RNA transcripts. This is often caused by multiple incomplete integrations of the transgene into the plant genome, often arranged as inverted repeats, which is a consequence of Agrobacterium-mediated transformation techniques [306,307,308,309]. These inverted repeats are transcribed to yield RNA that is self-complementary and therefore forms double-stranded structures that trigger the silencing mechanism [34,35,36].

RNA-dependent RNA-polymerases can also process aberrant single-stranded RNA, which can arise from fragmented integration of transgenes into the plant genome, to produce a double-stranded trigger of silencing [38,39].

Double-stranded RNA can also be formed if the transgene integrates into an endogenous gene in reverse orientation. In this case, the transgene promoter and the endogene promoter both drive transcription of the transgene sequence in opposite directions, resulting in the production of complimentary sense and antisense transcripts, which can pair to form double-stranded RNA. A recent study showed that transcriptional silencing of the transgene can also be triggered if the transgene promoter sequence is transcribed in sense orientation, driven by promoters present in flanking genomic sequences or in the bacterial DNA that is part of the transgene construct [310].

In addition, integration of a transgene into a densely methylated heterochromatic region of the genome can promote transcriptional silencing of the transgene, which often results in variegated expression. However, it has been reported that variations in transgene expression are more likely to be caused by the aforementioned fragmented and repeated integration of transgenes and not by position effects in *Arabidopsis thaliana* [311,312].

6.7.3 Assessment of evidence and implications for the practice

Self-silencing of transgenes appears to be a frequent outcome of plant transformation techniques. It is desirable to avoid or minimise this effect, which might be associated with hazards such as �off-target� silencing of unknown endogenous genes. In most cases expression of the transgene is the aim of genetic engineering and plant breeders would normally select against lines with silenced transgenes. Sometimes, however, the desired phenotype may be obtained with plants that, contrary to expectation, exhibit strong RNA silencing instead of expression of the transgene, which may even go unnoticed. This phenomenon is frequently found in virus resistant GM plants, which have often been designed to express a viral protein but were later found to be resistant because of the activation of antiviral RNA silencing [10-14].

The presence of siRNAs derived from the transgene and the methylation status of the transgene itself can be tested by well-established standard laboratory methods. It is reasonable to include such tests in the characterisation of GM plant lines that are intended to be released into the environment. The level of expression of a transgene can also be used as an indicator for the activation of silencing as much of the between line variation in transgene expression is likely to be due to silencing. Consequently any line that is expressing less than the maximal possible level of the transgene is likely to be exhibiting silencing to some extent. Conversely, no �off-target� silencing of endogenous genes or saturation of the silencing machinery have to be expected in plant lines where the transgene is not significantly affected by RNA silencing. As discussed above, several precautions can be taken to minimise silencing of a transgene.

Non-intentional RNA-silencing can also be caused by induced mutagenesis [124].� Because of the random character of this breeding technique it is not possible to analyse silencing of a target gene in this case.

6.7.4 Suggestions for future research

The fact that RNA silencing can be triggered by transgene expression in plants is well known. However, more research on the parameters that play a role in non-intentional triggering of RNA silencing, such as the characteristics of �aberrant� RNA, would help to further minimise non-intentional silencing of transgenes.

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Section 7 - Literature

1.��������� James, C. (2004) *Preview: Global Status of Commercialized Biotech/GM Crops: 2004. ISAAA Briefs No. 32* (ISAAA, Ithaca, NY).

2.��������� Napoli, C., Lemieux, C. & Jorgensen, R. A. (1990). Introduction of a chimeric chalcone synthase gene into Petunia results in reversible co-suppression of homologous genes *in trans*. *Plant Cell* **2**: 279-289

3.��������� van der Krol, A. R., Mur, L. A., Beld, M., Mol, J. N. & Stuitje, A. R. (1990). Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. *Plant Cell* **2**: 291-299

4.��������� Mol, J., van Blokland, R. & Kooter, J. (1991). More about co-suppression. *Trends Biotechnol* **9**: 182-183

5.��������� Van Blokland, R., Van der Geest, N., Mol, J. N. M. & Kooter, J. M. (1994). Transgene-mediated suppression of chalcone synthase expression in *Petunia hybrida* results from an increase in RNA turnover. *Plant J* **6**: 861-877

6.��������� de Carvalho, F., Gheysen, G., Kushnir, S., van Montagu, M., Inze, D. & Castresana, C. (1992). Suppression of beta-1,3-glucanase transgene expression in homozygous plants. *EMBO J* **11**: 2595-2602

7.��������� Baulcombe, D. C., English, J. J., Mueller, E. & Davenport, G. (1996) in *Mechanisms and applications of gene silencing.* 127-138 (Nottingham University Press, Nottingham).

8.��������� Lindbo, J. A. & Dougherty, W. G. (2005). Plant pathology and RNAi: a brief history. *Ann Rev Phytopathol* **43**: 191-204

9.��������� Lindbo, J. A., Silva-Roasales, L., Proebsting, W. M. & Dougherty, W. G. (1993). Induction of a Highly Specific Antiviral State in Transgenic Plants: Implications for Regulation of Gene Expression and Virus Resistance. *Plant Cell* **5**: 1749-1759

10.������� Pang, S. Z., Bock, J. H., Gonsalves, C., Slightom, J. L. & Gonsalves, D. (1994). Resistance of Transgenic Nicotiana benthamiana Plants to Tomato Spotted Wilt and Impatiens Necrotic Spot Tospoviruses - Evidence of Involvement of the N-Protein and N-gene RNA in Resistance. *Phytopathology* **84**: 243-249

11.������� Prins, M. & Goldbach, R. (1996). RNA-mediated virus resistance in transgenic plants. *Arch Virol* **141**: 2259-2276

12.������� Montgomery, M. K., Xu, S. Q. & Fire, A. (1998). RNA as a target of double-stranded RNA-mediated genetic interference in Caenorhabditis elegans. *Proc Natl Acad Sci USA* **95**: 15502-15507

13.������� Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E. & Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**: 806-811

14.������� Waterhouse, P. M., Graham, H. W. & Wang, M. B. (1998). Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. *Proc Natl Acad Sci USA* **95**: 13959-13964

15.������� Timmons, L. & Fire, A. (1998). Specific interference by ingested dsRNA. *Nature* **395**: 854

16.������� Wesley, S. V., Helliwell, C. A., Smith, N. A., Wang, M., Rouse, D. T., Liu, Q., Gooding, P. S. *et al.* (2001). Construct design for efficient, effective and high-throughput gene silencing in plants. *Plant J* **27**: 581-590

17.������� Zhen, W., ChangBin, C., YunYuan, X., RongXi, J., Ye, H., ZhiHong, X. & Kang, C. (2004). A practical vector for efficient knockdown of gene expression in rice (Oryza sativa L.). *Plant Mol Biol Rep* **22**: 409-417

18.������ Hunter, T., Hun, T., Jackson, R. J. & Robertson, H. D. (1975). The characteristics of inhibition of protein synthesis by double-stranded ribonucleic acid in reticulocyte lysates. *J Biol Chem* **250**: 409-417

19.������ Brummelkamp, T. R., Bernards, R. & Agami, R. (2002). A system for stable expression of short interfering RNAs in mammalian cells. *Science* **296**: 550-553

20.������ Ruiz, M. T., Voinnet, O. & Baulcombe, D. C. (1998). Initiation and maintenance of virus-induced gene silencing. *Plant� Cell* **10**: 937-946

21.������ Ratcliff, F., Martin-Hernandez, A. M. & Baulcombe, D. C. (2001). Tobacco rattle virus as a vector for analysis of gene function by silencing. *Plant J* **25**: 237-245

22.������ Hamilton, A. J., Voinnet, O., Chappell, L. & Baulcombe, D. C. (2002). Two classes of short interfering RNA in RNA silencing. *EMBO J* **21**: 4671-4679

23.������ Hamilton, A. J. & Baulcombe, D. C. (1999). A species of small antisense RNA in post-transcriptional gene silencing in plants. *Science* **286**: 950-952

24.������ Bernstein, E., Caudy, A. A., Hammond, S. M. & Hannon, G. J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409**: 363-366

25.������ Golden, T. A., Schauer, S. E., Lang, J. D., Pien, S., Mushegian, A. R., Grossniklaus, U., Meinke, D. W. *et al.* (2002). Short Integuments1/Suspensor1/Carpel Factory, a Dicer homolog, is a maternal effect gene required for embryo development in Arabidopsis. *Plant Physiol* **130**: 808-822

26.������ Carmell, M. A. & Hannon, G. J. (2004). RNase III enzymes and the initiation of gene silencing. *Nat Struct Mol Biol* **11**: 214-218

27.������ Qi, Y., Denli, A. M. & Hannon, G. J. (2005). Biochemical specialization within Arabidopsis RNA silencing pathways. *Mol Cell* **19**: 421-428

28.������ Xie, Z., Allen, E., Wilken, A. & Carrington, J. C. (2005). DICER-LIKE 4 functions in trans-acting small interfering RNA biogenesis and vegetative phase change in Arabidopsis thaliana. *Proc Natl Acad Sci USA* **102**: 12984-12989

29.������ Yoshikawa, M., Peragine, A., Park, M. Y. & Poethig, R. S. (2005). A pathway for the biogenesis of trans-acting siRNAs in Arabidopsis. *Genes Dev* **19**: 2164-2175

30.������ Park, W., Li, J., Song, R., Messing, J. & Chen, X. (2002). CARPEL FACTORY. a Dicer homolog, and HEN1, a novel protein, act in microRNA metabolism in *Arabidopsis thaliana*. *Curr Biol* **12**: 1484-1495

31.������ Kurihara, Y. & Watanabe, Y. (2004). Arabidopsis micro-RNA biogenesis through Dicer-like 1 protein functions. *Proc Natl Acad Sci USA* **101**: 12753-12758

32.������ Finnegan, E. J., Margis, R. & Waterhouse, P. M. (2003). Posttranscriptional gene silencing is not compromised in the *Arabidopsis CARPEL FACTORY* (*DICER-LIKE1*) mutant, a homolog of Dicer-1 from *Drosophila*. *Curr Biol* **13**: 236-240

33.������ Xie, Z., Johansen, L. K., Gustafson, A. M., Kasschau, K. D., Lellis, A. D., Zilberman, D., Jacobsen, S. E. *et al.* (2004). Genetic and Functional Diversification of Small RNA Pathways in Plants. *PLoS Biology* **2**: 642-652

34.������ Hutvagner, G. & Zamore, P. D. (2002). A microRNA in a multiple-turnover RNAi enzyme complex. *Science* **297**: 2056-2060

35.������ Baumberger, N. & Baulcombe, D. C. (2005). Arabidopsis ARGONAUTE1 is an RNA Slicer that selectively recruits microRNAs and short interfering RNAs. *Proc Natl Acad Sci USA* **102**: 11928-11933

36.������ Thomas, C. L., Jones, L., Baulcombe, D. C. & Maule, A. J. (2001). Size constraints for targeting post-transcriptional gene silencing and for RNA-directed methylation in *Nicotiana benthamiana* using a potato virus X vector. *Plant J* **25**: 417-425

37.������ Zamore, P. D., Tuschl, T., Sharp, P. A. & Bartel, D. P. (2000). RNAi: Double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* **101**: 25-33

38.������ Jorgensen, R., Snyder, C. & Jones, J. D. G. (1987). T-DNA is organized predominantly in inverted repeat structures in plants transformed with Agrobacterium tumefaciens C58 derivatives. *Mol Gen Genet* **207**: 471-477

39.������ Morino, K., Olsen, O. A. & Shimamoto, K. (2004). Silencing of the aleurone-specific Ltp2-gus gene in transgenic rice is reversed by transgene rearrangements and loss of aberrant transcripts. *Plant Cell Physiol* **45**: 1500-1508

40.������ Muskens, M. W. M., Vissers, A. P. A., Mol, J. N. M. & Kooter, J. M. (2000). Role of inverted DNA repeats in transcriptional and post- transcriptional gene silencing. *Plant Mol Biol* **43**: 243-260

41.������ Di Serio, F., Schob, H., Iglesias, A., Tarina, C., Bouldoires, E. & Meins, F. (2001). Sense- and antisense-mediated gene silencing in tobacco is inhibited by the same viral suppressors and is associated with accumulation of small RNAs. *Proc Natl Acad Sci USA* **98**: 6506-6510

42.������ Gazzani, S., Lawrenson, T., Woodward, C., Headon, D. & Sablowski, R. (2004). A link between mRNA turnover and RNA interference in Arabidopsis. *Science* **306**: 1046-1048

43.������ Wang, M. B. & Waterhouse, P. M. (2000). High-efficiency silencing of a beta-glucuronidase gene in rice is correlated with repetitive transgene structure but is independent of DNA methylation. *Plant Mol Biol* **43**: 67-82

44.������ Vaistij, F. E., Jones, L. & Baulcombe, D. C. (2002). Spreading of RNA targeting and DNA methylation in RNA silencing requires transcription of the target gene and a putative RNA-dependent RNA polymerase. *Plant Cell* **14**: 857-867

45.������ Klahre, U., Crete, P., Leuenberger, S. A., Iglesias, V. A. & Meins, F. (2002). High molecular weight RNAs and small interfering RNAs induce systemic posttranscriptional gene silencing in plants. *Proc Natl Acad Sci USA* **99**: 11981-11986

46.������ Voinnet, O., Vain, P., Angell, S. & Baulcombe, D. C. (1998). Systemic spread of sequence-specific transgene RNA degradation is initiated by localised introduction of ectopic promoterless DNA. *Cell* **95**: 177-187

47.������ Petersen, B. O. & Albrechtsen, M. (2005). Evidence implying only unprimed RdRP activity during transitive gene silencing in plants. *Plant Mol Biol* **58**: 575-583

48.������ Palauqui, J.-C., Elmayan, T., Pollien, J.-M. & Vaucheret, H. (1997). Systemic acquired silencing: transgene-specific post-transcriptional silencing is transmitted by grafting from silenced stocks to non-silenced scions. *EMBO J* **16**: 4738-4745

49.������ Voinnet, O. & Baulcombe, D. C. (1997). Systemic signalling in gene silencing. *Nature* **389**: 553

50.������ Fagard, M. & Vaucheret, H. (2000). Systemic silencing signal(s). *Plant Mol Biol* **43**: 285-293

51.������ Mlotshwa, S., Voinnet, O., Mette, M. F., Matzke, M., Vaucheret, H., Ding, S. W., Pruss, G. *et al.* (2002). RNA silencing and the mobile silencing signal. *Plant Cell* **14 Suppl**: S289-301

52.������ Yoo, B.-C., Kragler, F., Varkonyi-Gasic, E., Haywood, V., Archer-Evans, S., Lee, Y. M., Lough, T. J. *et al.* (2004). A Systemic Small RNA Signaling System in Plants. *Plant Cell* **16**: 1979-2000

53.������ Himber, C., Dunoyer, P., Moissiard, G., Ritzenthaler, C. & Voinnet, O. (2003). Transitivity-dependent and -independent cell-to-cell movement of RNA silencing. *EMBO J* **22**: 4523-4533

54.������ Schwach, F., Vaistij, F. E., Jones, L. & Baulcombe, D. (2005). An RNA-dependent RNA-polymerase prevents meristem invasion by Potato virus X and is required for the activity but not the production of a systemic silencing signal. *Plant Physiol* **138**: 1842-1852

55.������ Waterhouse, P. M., Wang, M. B. & Lough, T. (2001). Gene silencing as an adaptive defence against viruses. *Nature* **411**: 834-842

56.������ Voinnet, O., Lederer, C. & Baulcombe, D. C. (2000). A viral movement protein prevents spread of the gene silencing signal in *Nicotiana benthamiana*. *Cell* **103**: 157-167

57.������ Moissiard, G. & Voinnet, O. (2004). Viral suppression of RNA silencing in plants. *Mol Plant Pathol* **5**: 71-82

58.������ Ambros, V., Bartel, B., Bartel, D. P., Burge, C. B., Carrington, J. C., Chen, X., Dreyfuss, G. *et al.* (2003). A uniform system for microRNA annotation. *RNA* **9**: 277-279

59.������ Reinhart, B. J., Weinstein, E. G., Rhoades, M., Bartel, B. & Bartel, D. P. (2002). MicroRNAs in plants. *Genes Dev* **16**: 1616-1626

60.������ Papp, I., Mette, F., Aufsatz, W., Daxinger, L., Schauer, S. E., Ray, A., van der Winden, J. *et al.* (2003). Evidence for Nuclear Processing of Plant Micro RNA and Short Interfering RNA Precursors. *Plant Physiol* **132**: 1382-1390

61.������ Lim, L. P., Lau, N. C., Garrett-Engele, P., Grimson, A., Schelter, J. M., Castle, J., Bartel, D. P. *et al.* (2005). Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature* **433**: 769-773

62.������ Lee, R. C., Feinbaum, R. L. & Ambros, V. (1993). The C-Elegans Heterochronic Gene Lin-4 Encodes Small Rnas with Antisense Complementarity to Lin-14. *Cell* **75**: 843-854

63.������ Reinhart, B. J., Slack, F. J., Basson, M., Pasquinelli, A. E., Bettinger, J. C., Rougvie, A. E., Horvitz, H. R. *et al.* (2000). The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. *Nature* **403**: 901-906

64.������ Pasquinelli, A. F. & Ruvkun, G. (2002). Control of developmental timing by microRNAs and their targets. *Annu Rev Cell Dev Biol* **18**: 495-513

65.������ Ambros, V. (2003). MicroRNA pathways in flies and worms: growth, death, fat, stress, and timing. *Cell* **113**: 673-676

66.������ Bagga, S., Bracht, J., Hunter, S., Massirer, K., Holtz, J., Eachus, R. & Pasquinelli, A. E. (2005). Regulation by let-7 and lin-4 miRNAs Results in Target mRNA Degradation. *Cell* **122**

67.������ Bartel, D. P. (2004). MicroRNAs: Genomics, Biogenesis, Mechanism, and Function. *Cell* **116**: 281-297

68.������ Zeng, Y., Yi, R. & Cullen, B. R. (2003). MicroRNAs and smalll interfering RNAs can inhibit mRNA expression by similar mechanisms. *Proc Natl Acad Sci USA* **100**: 9779-9784

69.������ Jackson, A. L., Bartz, S. R., Schelter, J., Kobayshi, S. V., Burchard, J., Mao, M., Li, B. *et al.* (2003). Expression profiling reveals offf-target gene regulation by RNAi. *Nat Biotechnol* **21**: 635-637

70.������ Llave, C., Kasschau, K. D., Rector, M. A. & Carrington, J. C. (2002). Endogenous and silencing-associated small RNAs in plants. *Plant Cell* **14**: 1605-1619

71.������ Ingelbrecht, I., Van Houdt, H., Van Montagu, M. & Depicker, A. (1994). Posttranscriptional silencing of reporter transgenes in tobacco correlates with DNA methylation. *Proc Natl Acad Sci USA* **91**: 10502-10506

72.������ Mueller, E., Gilbert, J., Davenport, G., Brigneti, G. & Baulcombe, D. C. (1995). Homology-dependent resistance: transgenic virus resistance in plants related to homology-dependent gene silencing. *Plant J* **7**: 1001-1013

73.������ Niebel, F. C., Frendo, P., van Montagu, M. & Cornelissen, M. (1995). Post-Transcriptional Cosuppression of [beta]-1,3-Glucanase Genes Does Not Affect Accumulation of Transgene Nuclear mRNA. *Plant Cell* **7**: 347-358

74.������ Smith, H. A., Swaney, S. L., Parks, T. D., Wernsman, E. A. & Dougherty, W. G. (1994). Transgenic plant virus resistance mediated by untranslatable sense RNAs:� Expression, regulation, and fate of nonessential RNAs. *Plant Cell* **6**: 1441-1453

75.������ Jones, L., Ratcliff, F. & Baulcombe, D. C. (2001). RNA-directed transcriptional gene silencing in plants can be inherited independently of the RNA trigger and requires Met1 for maintenance. *Curr Biol* **11**: 747-757

76.������ Park, Y.-D., Papp, I., Moscone, E. A., Iglesias, V. A., Vaucheret, H., Matzke, A. J. M. & Matzke, M. A. (1996). Gene silencing mediated by promoter homology occurs at the level of transcription and results in meiotically heritable alterations in methylation and gene activity. *Plant J* **9**: 183-194

77.������ Neuhuber, F., Park, Y. D., Matzke, A. J. & Matzke, M. A. (1994). Susceptibility of transgene loci to homology-dependent gene silencing. *Mol Gen Genet* **244**: 230-241

78.������ Wakimoto, B. T. & Hearn, M. G. (1990). The effects of chromosome rearrangements on the expression of heterochromatic genes in chromosome 2L of Drosophila melanogaster. *Genetics* **125**: 141-154

79.������ Eberl, D. F., Duyf, B. J. & Hilliker, A. J. (1993). The role of heterochromatin in the expression of a heterochromatic gene, the rolled locus of Drosophila melanogaster. *Genetics* **134**: 277-292

80.������ Belyaeva, E. S., Demakova, O. V., Umbetova, G. H. & Zhimulev, I. F. (1993). Cytogenetic and molecular aspects of position-effect variegation in Drosophila melanogaster. V. Heterochromatin-associated protein HP1 appears in euchromatic chromosomal regions that are inactivated as a result of position-effect variegation. *Chromosoma* **102**: 583-590

81.������ Eissenberg, J. C. & Wallrath, L. L. (2003). Heterochromatin, Position Effects, and the Genetic Dissection of Chromatin. *Prog Nucleic Acid Res Mol Biol* **74**: 275-299

82.������ Tamaru, H. & Selker, E. U. (2001). A histone H3 methyltransferase controls DNA methylation in Neurospora crassa. *Nature* **414**: 277-283

83.������ Wassenegger, M., Heimes, S., Riedel, L. & Sanger, H. L. (1994). RNA-directed de novo methylation of genomic sequences in plants. *Cell* **76**: 567-576

84.������ Mette, M. F., Aufsatz, W., van der Winden, J., Matzke, M. A. & Matzke, A. J. M. (2000). Transcriptional silencing and promoter methylation triggered by double-stranded RNA. *EMBO J* **19**: 5194-5201

85.������ Wang, M. B., Wesley, S. V., Finnegan, E. J., Smith, N. A. & Waterhouse, P. M. (2001). Replicating satellite RNA induces sequence-specific DNA methylation and truncated transcripts in plants. *RNA* **7**: 16-28

86.������ Pelissier, T., Thalmeir, S., Kempe, D., Sanger, H. L. & Wassenegger, M. (1999). Heavy de novo methylation at symmetrical and non-symmetrical sites is a hallmark of RNA-directed DNA methylation. *Nucleic Acids Res* **27**: 1625-1634

87.������ Wada, Y., Ohya, H., Yamaguchi, Y., Koizumi, N. & Sano, H. (2003). Preferential de Novo Methylation of Cytosine Residues in Non-CpG Sequences by a

Domains Rearranged DNA Methyltransferase from Tobacco Plants. *J Biol Chem* **278**: 42386-42393

88.������ Cao, X. & Jacobsen, S. E. (2002). Locus-specific control of asymmetric and CpNpG methylation by the DRM and CMT3 methyltransferase genes. *Proc Natl Acad Sci USA* **99**: 16491-16498

89.������ Cao, X. F. & Jacobsen, S. E. (2002). Role of the Arabidopsis DRM methyltransferases in de novo DNA methylation and gene silencing. *Curr Biol* **12**: 1138-1144

90.������ Cao, X., Aufsatz, W., Zilberman, D., Mette, M. F., Huang, M. S., Matzke, M. & Jacobsen, S. E. (2003). Role of the *DRM* and *CMT3* Methyltransferases in RNA-Directed DNA Methylation. *Curr Biol* **13**: 2212-2217

91.������ Ekwall, K. (2004). The roles of histone modifications and small RNA in centromere function. *Chromosome Res* **12**: 535-542

92.������ Hall, I. M. & Grewal, S. I. (2003) in *RNAi: A Guide to Gene Silencing* (ed. Hannon, G. J.) 205-232 (Cold Spring Harbor Laboratory Press, Cold Spring Harbor).

93.������ Hall, I. M., Shankaranarayana, G. D., Noma, K., Ayoub, N., Cohen, A. & Grewal, S. I. (2002). Establishment and maintenance of a heterochromatin domain. *Science* **297**: 2232-2237

94.������ Volpe, T. A., Kidner, C., Hal, l. I. M., Teng, G., Grewal, S. I. & Martienssen, R. A. (2002). Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. *Science* **297**: 1833-1837

95.������ Noma, K., Sugiyama, T., Cam, H., Verdel, A., Zofall, M., Jia, S., Moazed, D. *et al.* (2004). RITS acts in cis to promote RNA interference-mediated transcriptional and post-transcriptional silencing. *Nat Genet* **36**: 1174-1180

96.������ Reinhart, B. J. & Bartel, D. P. (2002). Small RNAs correspond to centromere heterochromatic repeats. *Science* **297**: 1831

97.������ Sugiyama, T., Cam, H., Verdel, A., Moazed, D. & Grewal, S. I. S. (2005). RNA-dependent RNA polymerase is an essential component of a self-enforcing loop coupling heterochromatin assembly to siRNA production. *Proc Natl Acad Sci USA* **102**: 152-157

98.������ Verdel, A., Jia, S., Gerber, S., Sugiyama, T., Gygi, S., Grewal, S. I. S. & Moazed, D. (2004). RNAi-Mediated Targeting of Heterochromatin by the RITS Complex. *Science* **303**: 672-676

99.������ Baulcombe, D. (2004). RNA silencing in plants. *Nature* **431**: 356-363

100.���� Qi, Y. & Hannon, G. J. (2005). Uncovering RNAi mechanisms in plants: Biochemistry enters the foray. *FEBS Letters* **579**: 5899-5903

101.���� Lawrence, R. J. & Pikaard, C. S. (2003). Transgene-induced RNA interference: a strategy for overcoming gene redundancy in polyploids to generate loss-of-function mutations. *Plant J* **36**: 114-121

102.���� Segal, G., Song, R. T. & Messing, J. (2003). A new opaque variant of maize by a single dominant RNA-interference-inducing transgene. *Genetics* **165**: 387-397

103.���� Mette, M. F., Kanno, T., Aufsatz, W., Jakowitsch, J., van der Winden, J., Matzke, M. A. & Matzke, A. J. M. (2002). Endogenous viral sequences and their potential contribution to heritable virus resistance in plants. *EMBO J* **21**: 461-469

104.���� Hildebrandt, M. & Nellen, W. (1992). Differential antisense transcription from the Dictyostelium EB4 gene locus: implications on antisense-mediated regulation of mRNA stability. *Cell* **69**: 197-204

105.���� Matousek, J., Trnena, L., Oberhauser, R., Lichtenstein, C. P. & Nellen, W. (1994). dsRNA degrading nucleases are differentially expressed in tobacco anthers. *Biol*

Chem Hoppe Seyler **375**: 261-269

106.���� Zamecnik, P. C. & Stephenson, M. L. (1978). Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide. *Proc Natl Acad Sci USA* **75**: 280-284

107.���� Stephenson, M. L. & Zamecnik, P. C. (1978). Inhibition of Rous sarcoma viral RNA translation by a specific oligodeoxyribonucleotide. *Proc Natl Acad Sci USA* **75**: 285-288

108.���� Bourque, J. F. (1995). Antisense strategies for genetic manipulations in plants. *Plant Sci* **105**: 125-149

109.���� Smith, N. A., Singh, S. P., Wang, M. B., Stoutjesdijk, P. A., Green, A. G. & Waterhouse, P. M. (2000). Total silencing by intron-spliced hairpin RNAs. *Nature* **407**: 319-320

110.���� Sheehy, R. E., Kramer, M. & Hiatt, W. (1988). Reduction of polygalacturonase activity in tomato fruit by antisense RNA. *Proc Natl Acad Sci USA* **85**: 8805-8809

111.���� Sanders, R. A. & Hiatt, W. (2005). Tomato transgene structure and silencing. *Nat Biotechnol* **23**: 287-289

112.���� Medley, T. L. (1994). Availability of determination of nonregulated status for virus resistant squash. *Fed Regis* **59**: 64187-64189

113.���� Fuchs, M. & Gonsalves, D. (1995). Resistance of transgenic hybrid squash ZW-20 expressing the coat protein genes of zucchini yellow mosaic virus and watermelon mosaic virus 2 to mixed infections by both potyviruses. *Bio/Technology* **13**: 1466-1473

114.���� Tricoll, D. M., Carney, K. J., Russell, P. F., McMaster, J. R., Groff, D. W., Hadden, K. C., Himmel, P. T. *et al.* (1995). Field Evaluation of Transgenic Squash Containing Single or Multiple Virus Coat Protein Gene Constructs for Resistance to Cucumber Mosaic Virus, Watermelon Mosaic Virus 2, and Zucchini Yellow Mosaic Virus. *Bio/Technology* **13**: 1458-1465

115.���� Watson, J. M., Fusaro, A. F., Wang, M. & Waterhouse, P. M. (2005). RNA silencing platforms in plants. *FEBS Letters* **579**: 5982-5987

116.���� Kotting, O., Pusch, K., Tiessen, A., Geigenberger, P., Steup, M. & Ritte, G. (2005). Identification of a novel enzyme required for starch metabolism in Arabidopsis leaves. The phosphoglucan, water dikinase. *Plant Physiol* **137**: 242-252

117.���� Yin, Y. H., Vafeados, D., Tao, Y., Yoshida, S., Asami, T. & Chory, J. (2005). A new class of transcription factors mediates brassinosteroid-regulated gene expression in Arabidopsis. *Cell* **120**: 249-259

118.���� Higgins, J. D., Armstrong, S. J., Franklin, F. C. H. & Jones, G. H. (2004). The Arabidopsis MutS homolog AtMSH4 functions at an early step in recombination: evidence for two classes of recombination in Arabidopsis. *Genes Dev* **18**: 2557-2570

119.���� Farrona, S., Hurtado, L., Bowman, J. L. & Reyes, J. C. (2004). The Arabidopsis thaliana SNF2 homolog AtBRM controls shoot development and flowering. *Development* **131**: 4965-4975

120.���� Chuang, C.-H. & Meyerowitz, E. M. (2000). Specific and heritable genetic interference by double-stranded RNA in *Arabidopsis thaliana*. *Proc Natl Acad Sci USA* **97**: 4985-4990

121.���� Smith, N. A., Singh, S. P., Wang, M. B., Stoutjesdijk, P. A., Green, A. G. & Waterhouse, P. M. (2000). Total silencing by intron-spliced hairpin RNAs. *Nature* **407**: 319-320

122.���� Lin, R. C. & Wang, H. Y. (2004). Arabidopsis FHY3/FAR1 gene family and distinct roles of its members in light control of arabidopsis development. *Plant Physiol* **136**: 4010-4022

123.���� Mas, P., Alabadi, D., Yanovsky, M. J., Oyama, T. & Kay, S. A. (2003). Dual role of TOC1 in the control of circadian and photomorphogenic responses in Arabidopsis. *Plant Cell* **15**: 223-236

124.���� Kumar, D. & Klessig, D. F. (2003). High-affinity salicylic acid-binding protein 2 is required for plant innate immunity and has salicylic acid-stimulated lipase activity. *Proc Natl Acad Sci USA* **100**: 16101-16106

125.���� Helliwell, C. A., Wesley, S. V., Wielopolska, A. J. & Waterhouse, P. M. (2002). High-throughput vectors for efficient gene silencing in plants. *Funct Plant Biol* **29**: 1217-1225

126.���� Kusaba, M., Miyahara, K., Iida, S., Fukuoka, H., Takano, T., Sassa, H., Nishimura, M. *et al.* (2003). *Low glutelin content1*: a dominant mutation that suppresses the *Glutelin* multigene family via RNA silencing in rice. *Plant Cell* **15**: 1455-1467

127.���� Iida, K., Amano, E. & Nishio, T. (1993). A rice (Oryza-sativa L) mutant having a low content of glutelin and a high content of prolamine. *Theor Appl Genet* **87**: 374-378

128.���� Lu, P. Y., Xie, F. & Woodle, M. C. (2005). In vivo application of RNA interference: from functional genomics to therapeutics. *Adv Genet* **54**: 117-142

129.���� Lewis, D. L., Hagstrom, J. E., Loomis, A. G., Wolff, J. A. & Herweijer, H. (2002). Efficient delivery of siRNA for inhibition of gene expression in postnatal mice. *Nat Genet* **32**: 107-108

130.���� McCaffey, A. P., Meuse, L., Pham, T. T. T., Conklin, D. S., Hannon, G. J. & Kay, M. A. (2002). Gene expression: RNA interference in adult mice. *Nature* **418**: 38-39

131.���� Barik, S. (2005). Silence of the transcripts: RNA interference in medicine. *J Mol Med* **83**: 764-773

132.���� Barik, S. (2004). Development of gene-specific double-stranded RNA drugs. *Ann Med* **36**: 540-551

133.���� Whelan, J. (2005). First clinical data on RNAi. *Drug Discovery Today* **10**: 1014-1015

134.���� Zhou, Y. X., Chan, J. H., Chan, A. Y., Chak, R. K. F., Wong, E. Y. L., Chye, M. L., Peiris, J. S. M. *et al.* (2004). Transgenic plant-derived siRNAs can suppress propagation of influenza virus in mammalian cells. *FEBS Letters* **577**: 345-350

135.���� Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. & Tuschl, T. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**: 494-498

136.���� Elbashir, S. M., Lendeckel, W. & Tuschl, T. (2001). RNA interference is mediated by 21-and 22-nucleotide RNAs. *Genes Dev* **15**: 188-200

137.���� Elbashir, S. M., Martinez, J., Patkaniowska, A., Lendeckel, W. & Tuschl, T. (2001). Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. *EMBO J* **20**: 6877-6888

138.���� Scacheri, P. C., Rozenblatt-Rosen, O., Caplen, N. J., Wolfsberg, T. G., Umayam, L., Lee, J. C., Hughes, C. M. *et al.* (2004). Short interfering RNAs can induce unexpected and divergent changes in the levels of untargets proteins in mammalian cells. *Proc Natl Acad Sci USA* **101**: 1892-1897

139.���� Chi, J.-T., Chang, H. Y., Wang, N. N., Chang, D. S., Dunphy, N. & Brown, P. O. (2003). Genomewide view of gene silencing by small interfering RNAs. *Proc Natl Acad Sci USA* **100**: 6343-6346

140.���� Semizarov, D., Frost, L., Sarthy, A., Kroeger, P., Halbert, D. N. & Fesik, S. W. (2003). Specificity of short interfering RNA determined through gene expression signatures. *Proc Natl Acad Sci USA* **100**: 6347-6352

141.���� Persengiev, S. P., Zhu, X. & Green, M. R. (2004). Nonspecific, concentration-dependent stimulation and repression of mammalian gene expression by small interfering RNAs (siRNAs). *RNA* **10**: 12-18

142.���� Parizotto, E. A., Dunoyer, P., Rahm, N., Himber, C. & Voinnet, O. (2004). In vivo investigation of the transcription, processing, endonucleolytic activity, and functional relevance of the spatial distribution of a plant miRNA. *Genes Dev* **18**: 2237-2242

143.���� Tabara, H., Grishok, A. & Mello, C. C. (1998). RNAi in C-elegans: Soaking in the genome sequence. *Science* **282**: 430-431

144.���� Durand-Dubief, M., Kohl, L. & Bastin, P. (2003). Efficiency and specificity of RNA interference generated by intra- and intermolecular double stranded RNA in Trypanosoma brucei. *Molecular and Biochemical Parasitology* **129**: 11-21

145.���� Haley, B. & Zamore, P. D. (2004). Kinetic analysis of the RNAi enzyme complex. *Nat Struct Mol Biol* **11**: 599-606

146.���� Tang, G., Reinhart, B. J., Bartel, D. P. & Zamore, P. D. (2003). A biochemical framework for RNA silencing in plants. *Genes Dev* **17**: 49-63

147.���� Du, Q., Thonberg, H., Wang, J., Wahlestedt, C. & Liang, Z. (2005). A systematic analysis of the silencing effects of an active siRNA at all single-nucleotide mismatched target sites. *Nucleic Acids Res* **33**: 1671-1677

148.���� Holen, T., Moe, S. E., Sorbo, J. G., Meza, T. J., Ottersen, O. P. & Klungland, A. (2005). Tolerated wobble mutations in siRNAs decrease specificity, but can enhance activity in vivo. *Nucleic Acids Res* **33**: 4704-4710

149.���� Saxena, S., Jonsson, Z. O. & Dutta, A. (2003). Small RNAs with imperfect match to endogenous mRNA repress translation. Implications for off-target activity of small inhibitory RNA in mammalian cells. *J Biol Chem* **27**: 44312-44319

150.���� Reynolds, A., Leake, D., Boese, Q., Scaringe, S., Marshall, W. S. & Khvorova, A. (2004). Rational siRNA design for RNA interference. *Nat Biotechnol* **22**: 326-330

151.���� Jacque, J. M., Triques, K. & Stevenson, M. (2002). Modulation of HIV-1 replication by RNA interference. *Nature* **418**: 435-438

152.���� Holen, T., Amarzguioui, M., Wiiger, M. T., Babaie, E. & Prydz, H. (2002). Positional effects of short interfering RNAs targeting the human coagulation trigger Tissue Factor. *Nucleic Acids Res* **30**: 1757-1766

153.���� Gitlin, L., Stone, J. K. & Andino, R. (2005). Poliovirus Escape from RNA Interference: Short Interfering RNA-Target Recognition and Implications for Therapeutic Approaches. *J Virol* **79**: 1027-1035

154.���� Elbashir, S. M., Harborth, J., Weber, K. & Tuschl, T. (2002). Analysis of gene function in somatic mammalian cells using small interfering RNAs. *Methods* **26**: 199-213

155.���� Overhoff, M., Alken, M., Far, R. K. K., Lemaitre, M., Lebleu, B., Scakiel, G. & Robbins, I. (2005). Local RNA Target Structure Influences siRNA Efficacy: A Systematic Global Analysis. *J Mol Biol* **348**: 871-881

156.���� Schwab, R., Palatnik, J. F., Riester, M., Schommer, C., Schmid, M. & Weigel, D. (2005). Specific Effects of MicroRNAs on the Plant Transcriptome. *Dev Cell* **8**: 517-527

157.���� Archard, P., Herr, A., Baulcombe, D. C. & Harberd, N. P. (2004). Modulation of floral development by a gibberellin-regulated microRNA. *Development* **131**: 3357-3365

158.���� Aukerman, M. J. & Sakai, H. (2003). Regulation of Flowering Time and Floral Organ Identity by a MicroRNA and its *APETALA2*-Like Target Genes. *Plant Cell* **15**: 2730-2741

159.���� Chen, X. M. (2004). A microRNA as a translational repressor of APETALA2 in Arabidopsis flower development. *Science* **303**: 2022-2025

160.���� Juarez, M. T., Kul, J. S., Thomas, J., Heller, B. A. & Timmermans, M. C. P. (2004). microRNA-mediated repression of *rolled leaf1* specifies maize leaf polarity. *Nature* **428**: 84-88

161.���� Laufs, P., Peaucelle, A., Morin, H. & Traas, J. (2004). MicroRNA regulation of the CUC genes is required for boundary size control in *Arabidopsis* meristems. *Development* **131**: 4311-4322

162.���� Vaucheret, H., Vazquez, F., Crete, P. & Bartel, D. P. (2004). The action of ARGONAUTE1 in the miRNA pathway and its regulation by the miRNA pathway are crucial for plant development. *Genes Dev* **18**: 1187-1197

163.���� Xie, Z., Kasschau, K. D. & Carrington, J. C. (2003). Negative Feedback Regulation of *Dicer-Like1* in *Arabidopsis* by microRNA-Guided mRNA Degradation. *Curr Biol* **13**: 784-789

164.���� Rhoades, M. W., Reinhart, B. J., Lim, L. P., Burge, C. B., Bartel, B. & Bartel, D. P. (2002). Prediction of plant microRNA targets. *Cell* **110**: 513-520

165.���� Salehuzzaman, S. N. I. M., Jacobsen, E. & Visser, R. G. F. (1993). Isolation and characterization of a cDNA encoding granule-bound starch synthase in cassava (*Manihot esculenta Crantz*) and its antisense expression in potato. *Plant Mol Biol* **23**: 947-962

166.���� Visser, R. G., Somhorst, I., Kuipers, G. J., Ruys, N. J., Feenstra, W. J. & Jacobsen, E. (1991). Inhibition of the expression of the gene for granule-bound starch synthase in potato by antisense constructs. *Mol Gen Genet* **225**: 289-296

167.���� Visser, R. G. F., Kuipers, A. G. J. & Jacobsen, E. (1996) in *Mechanisms and aplications of gene silencing* (eds. Grierson, D., Lycett, G. W. & Tucker, G. A.) 97-104 (Nottingham University Press, Nottingham).

168.���� Kuipers, A. G. J., Vreem, J. T. M., Meyer, H., Jacobsen, E., Feenstra, W. J. & Visser, R. G. F. (1992). Field evaluation of antisense RNA mediated inhibition of GBSS gene expression in potato. *Euphytica* **59**: 83-91

169.���� Kuipers, A. G. J., Jacobsen, E. & Visser, R. G. F. (1994). Formation and deposition of amylose in the potato tuber starch granule are affected by the reduction of granule-bound starch synthase gene expression. *Plant Cell* **6**: 43-52

170.���� Kuipers, A. G. J., Soppe, W. J., Jacobsen, E. & Visser, R. G. F. (1994). Field evaluation of transgenic potato plants expressing an antisense granule-bound starch synthase gene: increase of the antisense effect during tuber growth. *Plant Mol Biol* **26**: 1759-1773

171.���� Kuipers, A. G. J., Soppe, W. J., Jacobsen, E. & Visser, R. G. F. (1995). Factors affecting the inhibition by antisense RNA of granule-bound starch synthase gene expression in potato. *Mol Gen Genet* **246**: 745-755

172.���� Visser, R. G. F.(1989) (University of Groningen, Groningen, Netherlands).

173.���� de Carvalho Niebel, F., Frendo, P., Van Montagu, M. & Cornelissen, M. (1995). Post-transcriptional cosuppression of b-1,3-glucanase genes does not affect accumulation of transgene nuclear mRNA. *Plant Cell* **7**: 347-358

174.���� Thierry, D. & Vaucheret, H. (1996). Sequence homology requirements for transcriptional silencing of 35S transgenes and post-transcriptional silencing of nitrite reductase (trans)genes by the tobacco 271 locus. *Plant Mol Biol* **32**: 1075-1083

175.���� Angenent, G. C., Franken, J., Busscher, M., Colombo, L. & van Tunen, A. J. (1993). Petal and stamen formation in petunia is regulated by the homeotic gene fbp1. *Plant J* **4**: 101-112

176.���� Kunz, C., Schob, H., Leubner-Metzger, G., Glazov, E. & Meins Jr, F. (2001). b-1,3-Glucanase and chitinase transgenes in hybrids show distinctive and independent patterns of posttranscriptional gene silencing. *Planta* **212**: 243-249

177.���� Kunz, C., Schob, H., Stam, M., Kooter, J. M. & Meins, F. J. (1996). Developmentally regulated silencing and reactivation of tobacco chitinase transgene expression. *Plant J* **10**: 437-450

178.���� Pawloski, L. C., Deal, R. B., McKinney, E. C., Burgos-Rivera, B. & Meagher, R. B. (2005). Inverted Repeat PCR for the Rapid Assembly of Constructs to Induce RNA Interference. *Plant Cell Physiol* **Advance Access published online on 24/08/05**

179.���� Ifuku, K., Yamamoto, Y. & Sato, F. (2003). Specific RNA interference in psbP genes encoded by a multigene family in Nicotiana tabacum with a short 3'-untranslated sequence. *Biosci Biotechnol Biochem* **67**: 107-113

180.���� Fukusaki, E., Kawasaki, K., Kajiyama, S., An, C. I., Suzuki, K., Tanaka, Y. & A., K. (2004). Flower color modulations of Torenia hybrida by downregulation of chalcone synthase genes with RNA interfere nce. *J Biotechnol* **111**: 229-240

181.���� Ogita, S., Uefuji, H., Morimoto, M. & Sano, H. (2004). Application of RNAi to confirm theobromine as the major intermediate for caffeine biosynthesis in coffee plants with potential for construction of decaffeinated varieties. *Plant Mol Biol* **54**: 931-941

182.���� Han, Y. H., Grierson, D. & Han, Y. H. (2002). Relationship between small antisense RNAs and aberrant RNAs associated with sense transgene mediated gene silencing in tomato. *Plant J* **29**: 509-519

183.���� Doench, J. G., Petersen, C. P. & Sharp, P. A. (2003). siRNAs can function as miRNAs. *Genes Dev* **17**: 438-442

184.���� Herman, E. M., Helm, R. M., Jung, R. & Kinney, A. J. (2003). Genetic modification removes an immunodominant allergen from soybean. *Plant Physiol* **132**: 36-43

185.���� Lin, S. Y., Johnson, S. M., Abraham, M., Vella, M. C., Pasquinelli, A., Gamberi, C., Gottlieb, E. *et al.* (2003). The C elegans hunchback homolog, hbl-1, controls temporal patterning and is a probable microRNA target. *Dev Cell* **4**: 639-650

186.���� Brennecke, J., Hipfner, D. R., Stark, A., Russell, R. B. & Cohen, S. M. (2003). *bantam* Encodes a Developmentally Regulated microRNA that Controls Cell Proliferation and Regulates the Proapoptotic Gene *hid* in *Drosophila*. *Cell* **113**: 25-36

187.���� Wightman, B., Ha, I. & Ruvkun, G. (1993). Posttranscriptional Regulation of the Heterochronic Gene Lin-14 by Lin-4 Mediates Temporal Pattern-Formation in C-Elegans. *Cell* **75**: 855-862

188.���� Moss, E. G., Lee, R. C. & Ambros, V. (1997). The cold shock domain protein LIN-28 controls developmental timing in C. elegans and is regulated by the lin-4 RNA. *Cell* **88**: 637-646

189.���� Slack, F. J., Basson, M., Liu, Z., Ambros, V., Horvitz, H. R. & Ruvkun, G. (2000). The lin-41 RBCC gene acts in the C. elegans heterochronic pathway between the let-7 regulatory RNA and the LIN-29 transcription factor. *Mol Cell* **5**: 659-669

190.���� Abrahante, J. E., Dau, l. A. L., Li, M., Volk, M. L., Tennessen, J. M., Miller, E. A. & Rougvie, A. E. (2003). The Caenorhabditis elegans hunchback-like gene lin-57/hbl-1 controls developmental time and is regulated by microRNAs. *Dev Cell* **4**: 625-637

191.���� Banerjee, D. & Slack, F. (2002). Control of developmental timing by small temporal RNAs: a paradigm for RNA-mediated regulation of gene expression.

Bioessays **24**: 119-129

192.���� Lu, S., Shi, R., Tsao, C. C., Yi, X., Li, L. & Chiang, V. L. (2004). RNA silencing in plants by the expression of siRNA duplexes. *Nucleic Acids Res* **32**: e171

193.���� Qiu, S., Adema, C. M. & Lane, T. (2005). A computational study of off-target effects of RNA interference. *Nucleic Acids Res* **33**: 1834-1847

194.���� Korf, I., Yandell, M., Bedell & J., F. (2003) *An essential guide to the basic local alignment tool* (O'Reilly, Bejing).

195.���� Snove, O. & Holen, T. (2004). Many commonly used siRNAs risk off-target activity. *Biochem Biophys Res Commun* **319**: 256-263

196.���� Arziman, Z., Horn, T. & Boutros, M. (2005). E-RNAi: a web application to design optimized RNAi constructs. *Nucleic Acids Res* **33**: 582-588

197.���� Naito, Y., Yamada, T., Matsumiya, T., Ui-Tei, K., Saigo, K. & Morishita, S. (2005). dsCheck: highly sensitive off-target search software for double-stranded RNA-mediated RNA interference. *Nucleic Acids Res* **33**: 589-591

198.���� Kuiper, H. A., Kleter, G. A., Noteborn, H. P. & Kok, E. J. (2002). Substantial equivalence--an appropriate paradigm for the safety assessment of genetically modified foods? *Toxicology* **181-182**: 427-431

199.���� Kok, E. J. & Kuiper, H. A. (2003). Comparative safety assessment for biotech crops. *Trends Biotechnol* **21**: 439-444

200.���� Catchpole, G. S., Beckmann, M., Enot, D. P., Mondhe, M., Zywicki, B., Taylor, J., Hardy, N. *et al.* (2005). Hierarchical metabolomics demonstrates substantial compositional similarity between genetically modified and conventional potato crops. *Proc Natl Acad Sci USA* **102**: 14458-14462

201.���� Siolas, D., Lerner, C., Burchard, J., Ge, W., Linsley, P. S., Paddison, P. J., Hannon, G. J. *et al.* (2005). Synthetic shRNAs as potent RNAi triggers. *Nat Biotechnol* **23**: 227-231

202.���� Davuluri, G. R., van Tuinen, A., Fraser, P. D., Manfredonia, A., Newman, R., Burgess, D., Brummell, D. A. *et al.* (2005). Fruit-specific RNAi-mediated suppression of DET1 enhances carotenoid and flavonoid content in tomatoes. *Nat Biotechnol* **23**: 890-895

203.���� Byzova, M., Verduyn, C., De Brouwer, D. & De Block, M. (2004). Transforming petals into sepaloid organs in Arabidopsis and oilseed rape: implementation of the hairpin RNA-mediated gene silencing technology in an organ-specific manner. *Planta* **218**: 379-387

204.���� Liu, Q., Singh, S. P. & Green, A. G. (2002). High-stearic and high-oleic cottonseed oils produced by hairpin RNA-mediated post-transcriptional gene silencing. *Plant Physiol* **129**: 1732-1743

205.���� Stoutjesdijk, P. A., Singh, S. P., Liu, Q., Hurlstone, C. J., Waterhouse, P. A. & Green, A. G. (2002). hpRNA-Mediated Targeting of the Arabidopsis FAD2 Gene Gives Highly Efficient and Stable Silencing. *Plant Physiol* **129**: 1723-1731

206.���� Wilkinson, J. E., Lindsey, K. & Twell, D. (1998). Antisense-mediated suppression of transgene expression targeted specifically to pollen. *Journal of Experimental Botany* **49**: 1481-1490

207.���� Migge, A. & Becker, T. W. (2000). Greenhouse-grown conditionally lethal tobacco plants obtained by expression of plastidic glutamine synthetase antisense RNA may contribute to biological safety. *Plant Sci* **153**: 107-112

208.���� Mitsuhara, I., Shirasawa-Seo, N., Iwai, T., Nakamura, S., Honkura, R. & Ohashi, Y. (2002). Release from post-transcriptional gene silencing by cell proliferation in transgenic tobacco plants: possible mechanism for noninheritance of the silencing. *Genetics* **160**: 343-352

209.���� Que, Q. D., Wang, H. Y., English, J. J. & Jorgensen, R. A. (1997). The frequency and degree of cosuppression by sense chalcone synthase transgenes are dependent on transgene promoter strength and are reduced by premature nonsense codons in the transgene coding sequence. *Plant Cell* **9**: 1357-1368

210.���� Qin, H., Dong, Y. & von Arnim, A. G. (2003). Epigenetic interactions between Arabidopsis transgenes: characterization in light of transgene integration sites. *Plant Mol Biol* **52**: 217-231

211.���� Palauqui, J. C., De Borne, F. D., Elmayan, T., Crete, P., Charles, C. & Vaucheret, H. (1996). Frequencies, Timing, and Spatial Patterns Of Co-Suppression Of Nitrate Reductase and Nitrite Reductase In Transgenic Tobacco Plants. *Plant Physiol* **112**: 1447-1456

212.���� Elmayan, T. & Vaucheret, H. (1996). Expression of single copies of a strongly expressed *35S* transgene can be silenced post-transcriptionally. *Plant Journal* **9**: 787-797

213.���� Palauqui, J.-C. & Vaucheret, H. (1995). Field trial analysis of nitrate reductase co-suppression - a comparative-study of 38 combinations of transgene loci. *Plant Mol Biol* **29**: 149-159

214.���� Dorlhac de Borne, F. D., Vincentz, M., Chupeau, Y. & Vaucheret, H. (1994). Co-suppression of nitrate reductase host genes and transgenes in transgenic tobacco plants. *Mol Gen Genet* **243**: 613-621

215.���� Hipskind, J. D. & Paiva, N. L. (2000). Constitutive accumulation of a resveratrol-glucoside in transgenic alfalfa increases resistance to Phoma medicaginis. *Mol Plant Microbe Interact* **13**: 551-562

216.���� Battraw, M. J. & Hall, T. C. (1990). Histochemical analysis of CaMV 35S promoter-beta-glucuronidase gene expression in transgenic rice plants. *Plant Mol Biol* **15**: 527-538

217.���� Sunilkumar, G., Mohr, L., Lopata-Finch, E., Emani, C. & Rathore, K. S. (2002). Developmental and tissue-specific expression of CaMV 35S promoter in cotton as revealed by GFP. *Plant Mol Biol* **50**: 463-474

218.���� Harper, B. K. & Stewart jr, C. N. (2000). Patterns of Green Fluorescent Protein Expression in Transgenic Plants. *Plant Mol Biol Rep* **18**: 141a-141i

219.���� Dong, J. Z., Yang, M. Z., Jia, S. R. & Chua, N. H. (1991). Transformation of Melon (Cucumis melo L.) and Expression from the Cauliflower Mosaic Virus 35S Promoter in Transgenic Melon Plants. *Nat Biotechnol* **9**: 858-863

220.���� Andika, I. B., Kondo, H. & Tamada, T. (2005). Evidence that RNA silencing-mediated resistance to Beet necrotic yellow vein virus is less effective in roots than in leaves. *Mol Plant Microbe Interact* **18**: 194-204

221.���� Aronen, T., Hohtola, A., Laukkanen, H. & Haggman, H. (1995). Seasonal changes in the transient expression of a 35S CaMV-GUS gene construct introduced into Scots pine buds. *Tree Physiol* **15**: 65-70

222.���� Correa, R. L., Gomes, L. L., Margis, R. & Vaslin, M. F. S. (2004). Suppression of post-transcriptional gene silencing by callus induction and virus infection reveals the existence of aberrant RNAs. *Plant Sci* **167**: 159-164

223.���� Finnegan, J. & McElroy, D. (1994). Transgene inactivation:� Plants fight back! *Bio/Technology* **12**: 883-888

224.���� Kilby, N. J., Leyser, H. M. & Furner, I. J. (1992). Promoter methylation and progressive transgene inactivation in Arabidopsis. *Plant Mol Biol* **20**: 103-112

225.���� Kumpatla, S. P. & Hall, T. C. (1998). Recurrent onset of epigenetic silencing in rice harboring a multi-copy transgene. *Plant J* **14**: 129-135

226.���� Meng, L., Bregitzer, P., Zhang, S. B. & Lemaux, P. G. (2003). Methylation of the exon/intron region in the Ubi1 promoter complex correlates with transgene silencing in barley. *Plant Mol Biol* **53**: 327-340

227.���� Kloti, A., He, X., Potrykus, I., Hohn, T. & Futterer, J. (2002). Tissue-specific silencing of a transgene in rice. *Proc Natl Acad Sci U S A* **99**: 10881-10886

228.���� Mette, M. F., van der Winden, J., Matzke, M. A. & Matzke, A. J. M. (1999). Production of aberrant promoter transcripts contributes to methylation and silencing of unlinked homologous promoters in trans. *EMBO J* **18**: 241-248

229.���� Beclin, C., Boutet, S., Waterhouse, P. & Vaucheret, H. (2002). A branched pathway for transgene-induced RNA silencing in plants. *Curr Biol* **12**: 684-688

230.���� Aufsatz, W., Mette, M. F., van der Winden, J., Matzke, A. J. M. & Matzke, M. (2002). RNA-directed DNA methylation in *Arabidopsis*. *Proc Natl Acad Sci USA* **99**: 16499-16506

231.���� Jones, L., Hamilton, A. J., Voinnet, O., Thomas, C. L., Maule, A. J. & Baulcombe, D. C. (1999). RNA-DNA interactions and DNA methylation in post-transcriptional gene silencing. *Plant Cell* **11**: 2291-2302

232.���� Kunz, C., Narangajavana, J., Jakowitsch, J. Y., Park, Y. D., Delon, T. R., Kovarik, R., Koukalov�, B. *et al.* (2003). Studies on the effects of a flanking repetitive sequence on the expression of single copy transgenes in Nicotiana sylvestris and in N. sylvestris-N. tomentosiformis hybrids. *Plant Mol Biol* **52**: 203-215

233.���� Wang, M. B., Wesley, S. V., Finnegan, E. J., Smith, N. A. & Waterhouse, P. M. (2001). Replicating satellite RNA induces sequence-specific DNA methylation and truncated transcripts in plants. *RNA* **7**: 16-28

234.���� Vogt, U., Pelissier, T., Putz, A., Razvi, F., Fischer, R. & Wassenegger, M. (2004). Viroid-induced RNA silencing of GFP-viroid fusion transgenes does not induce extensive spreading of methylation or transitive silencing. *Plant J* **38**: 107-118

235.���� ten Lohuis, M., Mueller, A., Heidmann, I., Niedenhof, I. & Meyer, P. (1995). A repetitive DNA fragment carrying a hot spot for de novo DNA methylation enhances expression variegation in tobacco and petunia. *Plant J* **8**: 919-932

236.���� Arnaud, P., Goubely, C., Pelissier, T. & Deragon, J. M. (2000). SINE Retroposons Can Be Used In Vivo as Nucleation Centers for De Novo Methylation. *Mol Cell Biol* **20**: 3434-3441

237.���� Korth, K. L., Blount, J. W., Chen, F., Rasmussen, S., Lamb, C. & Dixon, R. A. (2001). Changes in phenylpropanoid metabolites associated with homology-dependent silencing of phenylalanine ammonia-lyase and its somatic reversion in tobacco. *Physiologia Plantarum* **111**: 137-143

238.���� Balandin, T. & Castresana, C. (1997). Silencing of a beta-1,3-glucanase transgene is overcome during seed formation. *Plant Mol Biol* **34**: 125-137

239.���� Dehio, C. & Schell, J. (1994). Identification of plant genetic loci involved in posttranscriptional mechanism for meiotically reversible transgene silencing. *Proc Natl Acad Sci USA* **91**: 5538-5542

240.���� Hart, C. M., Fischer, B., Neuhaus, J. M. & Meins, F. J. (1992). Regulated inactivation of homologous gene expression in transgenic Nicotiana sylvestris plants containing a defense-related tobacco chitinase gene. *Mol Gen Genet* **235**: 179-188

241.���� Fojtova, M., Van Houdt, H., Depicker, A. & Kovarik, A. (2003). Epigenetic Switch from Posttranscriptional to Transcriptional Silencing Is Correlated with Promoter Hypermethylation. *Plant Physiol* **133**: 1240-1250

242.���� Liu, Y. S., Levin, J. S., Murray, J. S., Wernsman, E. A. & Weissinger, A. K. (2003). A multi-generation analysis of the stability of transgenic virus resistance in doubled-haploid tobacco lines. *Molecular Breeding* **12**: 145-156

243.���� Vaucheret, H., Kronenberger, J., Lepingle, A., Vilaine, F., Boutin, J. P. & Caboche, M. (1992). Inhibition of tobacco nitrite reductase activity by expression of antisense RNA. *Plant J* **2**: 559-569

244.���� Planchet, E., Gupta, K. J., Sonoda, M. & Kaiser, W. M. (2005). Nitric oxide emission from tobacco leaves and cell suspensions: rate limiting factors and evidence for the involvement of mitochondrial electron transport. *Plant J* **41**: 732-743

245.���� Chabannes, M., Barakate, A., Lapierre, C., Marita, J. M., Ralph, J., Pean, M., Danoun, S. *et al.* (2001). Strong decrease in lignin content without significant alteration of plant development is induced by simultaneous down-regulation of cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) in tobacco plants. *Plant J* **28**: 257-270

246.���� Pincon, G., Chabannes, M., Lapierre, C., Pollet, B., Ruel, K., Joseleau, J. P., Boudet, A. M. *et al.* (2001). Simultaneous down-regulation of caffeic/5-hydroxy ferulic acid-O-methyltransferase I and cinnamoyl-coenzyme a reductase in the progeny from a cross between tobacco lines homozygous for each transgene. Consequences for plant development and lignin synthesis. *Plant Physiol* **126**: 145-155

247.���� Abbott, J. C., Barakate, A., Pincon, G., Legrand, M., Lapierre, C., Mila, I., Schuch, W. *et al.* (2002). Simultaneous suppression of multiple genes by single transgenes. Downregulation of three unrelated lignin biosynthetic genes in tobacco. *Plant Physiol* **128**: 844-853

248.���� Vance, V. B. (1991). Replication of Potato Virus-X Rna Is Altered in Coinfections with Potato Virus-Y. *Virology* **182**: 486-494

249.���� Pruss, G., Ge, X., Shi, X. M., Carrington, J. C. & Bowman Vance, V. (1997). Plant viral synergism: the potyviral genome encodes a broad-range pathogenicity enhancer that transactivates replication of heterologous viruses. *Plant Cell* **9**: 859-868

250.���� Anandalakshmi, R., Pruss, G. J., Ge, X., Marathe, R., Smith, T. H. & Vance, V. B. (1998). A viral suppressor of gene silencing in plants. *Proc Natl Acad Sci USA* **95**: 13079-13084

251.���� Brigneti, G., Voinnet, O., Li, W. X., Ji, L. H., Ding, S. W. & Baulcombe, D. C. (1998). Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. *EMBO J* **17**: 6739-6746

252.���� Kasschau, K. D. & Carrington, J. C. (1998). A counterdefensive strategy of plant viruses: suppression of posttranscriptional gene silencing. *Cell* **95**: 461-470

253.���� Teycheney, P. Y. & Tepfer, M. (2001). Virus-specific spatial differences in the interference with silencing of the chs-A gene in non-transgenic petunia. *J Gen Virol* **82**: 1239-1243

254.���� Senda, M., Masuta, C., Ohnishi, S., Goto, K., Kasai, A., Sano, T., Hong, J. S. *et al.* (2004). Patterning of Virus-Infected Glycine max Seed Coat Is Associated with Suppression of Endogenous Silencing of Chalcone Synthase Genes. *Plant Cell* **16**: 807-818

255.���� Tuteja, J. H., Clough, S. J., Chan, W. C. & Vodkin, L. O. (2004). Tissue-Specific Gene Silencing Mediated by a Naturally Occurring Chalcone Synthase Gene Cluster in Glycine max. *Plant Cell* **16**: 819-835

256.���� Llave, C., Kasschau, K. D. & Carrington, J. C. (2000). Virus-encoded suppressor of posttranscriptional gene silencing targets a maintenance step in the silencing pathway. *Proc Natl Acad Sci USA* **97**: 13401-13406

257.���� Mitter, N., Sulistyowati, E., Graham, M. W. & Dietzgen, R. G. (2001). Suppression of gene silencing: a threat to virus-resistant transgenic plants? *Trends Plant Sci* **6**: 246-247

258.���� Di Serio, F., Rubino, L., Russo, M. & Martelli, G. P. (2002). Homology-dependent virus resistance against Cymbidium ringspot virus is inhibited by post-transcriptional gene silencing suppressor viruses. *Journal of Plant Pathology* **84**: 121-124

259.���� Mitter, N., Sulistyowati, E. & Dietzgen, R. G. (2003). Cucumber mosaic virus infection transiently breaks dsRNA-Induced Transgenic immunity to Potato virus Y in tobacco. *Mol Plant Microbe Interact* **16**: 936-944

260.���� Savenkov, E. I. & Valkonen, J. P. T. (2001). Coat protein gene-mediated resistance to Potato virus A in transgenic plants is suppressed following infection with another potyvirus. *J Gen Virol* **82**: 2275-2278

261.���� Al-Kaff, N. S., Kreike, M. M., Covey, S. N., Pitcher, R., Page, A. M. & Dale, P. J. (2000). Plants rendered herbicide-susceptible by cauliflower mosaic virus-elicited suppression of a 35S promoter-regulated transgene. *Nat Biotechnol* **18**: 995-999

262.���� Escaler, M., Aranda, M. A., Thomas, C. L. & Maule, A. J. (2000). Pea embryonic tissues show common responses to the replication of a wide range of viruses. *Virology* **267**: 318-325

263.���� Aranda, M. A., Escaler, M., Wang, D. & Maule, A. J. (1996). Induction of HSP70 and polyubiquitin expression associated with plant virus replication. *Proc Natl Acad Sci USA* **93**: 15289-15293

264.���� Escaler, M., Aranda, M. A., Roberts, I. M., Thomas, C. L. & Maule, A. J. (2000). A comparison between virus replication and abiotic stress (heat) as modifiers of host gene expression in pea. *Mol Plant Pathol* **1**: 159-167

265.���� Taliansky, M. E., Kim, S. H., Mayo, M. A., Kalinina, N. O., Fraser, G., McGeachy, K. D. & Barker, H. (2004). Escape of a plant virus from amplicon-mediated RNA silencing is associated with biotic or abiotic stress. *Plant J* **39**: 194-205

266.���� English, J. J. & Baulcombe, D. C. (1997). The influence of small changes in transgene transcription on homology-dependent virus resistance and gene silencing. *Plant J* **12**: 1311-1318

267.���� Kovarik, A., Koukalova, B., Bezdek, M. & Opatrny, Z. (1997). Hypermethylation of tobacco heterochromatic loci in response to osmotic stress. *Theor Appl Genet* **95**: 301-306

268.���� Schmitt, F., Oakeley, E. J. & Jost, J. P. (1997). Antibiotics Induce Genome-ide Hypermethylation in Cultured *Nicotiana tabacum*� Plants. *J Biol Chem* **272**: 1534-1540

269.���� Teerawanichpan, P., Chandrasekharan, M. B., Jiang, Y. M., Narangajavana, J. & Hall, T. C. (2004). Characterization of two rice DNA methyltransferase genes and RNAi-mediated reactivation of a silenced transgene in rice callus. *Planta* **218**: 337-349

270.���� Managanaris, G. A., Economou, A. S., Boubourakas, I. N. & Katis, N. I. (2003). Elimination of PPV and PNRSV through thermotherapy and meristem-tip culture in nectarine. *Plant Cell Rep* **22**: 195-200

271.���� Szittya, G., Silhavy, D., Molnar, A., Havelda, Z., Lovas, A., Lakatos, L., Banfalvi, Z. *et al.* (2003). Low temperature inhibits RNA silencing-mediated defence by the control of siRNA generation. *EMBO J* **22**: 633-640

272.���� Havelda, Z., Hornyik, C., V�l�czi, A. & Burgy�n, J. (2005). Defective Interfering RNA Hinders the Activity of a Tombusvirus-Encoded Posttranscriptional Gene Silencing Suppressor. *J Virol* **79**: 450-457

273.���� Chellappan, P., Vanitharani, R., Ogbe, F. & Fauquet, C. (2005). Effect of Temperature on Geminivirus-Induced RNA Silencing in Plants. *Plant Physiol* **138**: 1828-1841

274.���� Finnegan, E. J., Genger, R. K., Peacock, W. J. & Dennis, E. S. (1998). DNA methylation in plants. *Annu Rev Plant Physiol Plant Mol Biol* **49**: 223-247

275.���� Meyer, P., Linn, F., Heidmann, I., Meyer, H., Niedenhof, I. & Saedler, H. (1992). Endogenous and Environmental-Factors Influence 35s Promoter Methylation of a Maize A1 Gene Construct in Transgenic Petunia and Its Color Phenotype. *Mol Gen Genet* **231**: 345-352

276.���� Nielsen, K., Deroles, S. C., Markham, K. R., Bradley, M. J., Podivinsky, E. & Manson, D. (2002). Antisense flavonol synthase alters copigmentation and flower color in lisianthus. *Molecular Breeding* **9**: 217-229

277.���� Hily, J. M., Scorza, R., Malinowski, T., Zawadzka, B. & Ravelonandro, M. (2004). Stability of gene silencing-based resistance to Plum pox virus in transgenic plum (Prunus domestica L.) under field conditions. *Transgenic Res* **13**: 427-436

278.���� LiJen, L., IChun, P., YuanLi, C., YauHeiu, H., WenHuei, C. & MingTsair, C. (2004). Transgene silencing in Phalaenopsis expressing the coat protein of Cymbidium Mosaic Virus is a manifestation of RNA-mediated resistance. *Molecular Breeding* **13**: 229-242

279.���� Levin, J. S., Thompson, W. F., Csinos, A. S., Stephenson, M. G. & Weissinger, A. K. (2005). Matrix attachment regions increase the efficiency and stability of RNA-mediated resistance to tomato spotted wilt virus in transgenic tobacco. *Transgenic Res* **14**: 193-206

280.���� Ferreira, S. A., Pitz, K. Y., Manshardt, R., Zee, F., Fitch, M. & Gonsalves, D. (2001). Virus Coat Protein Transgenic Papaya Provides Practical Control of Papaya ringspot virus in Hawaii. *Plant Dis.* **86**: 101-105

281.���� Pilate, G., Guiney, E., Holt, K., Petit-Conil, M., Lapierre, C., Leple, J. C., Pollet, B. *et al.* (2002). Field and pulping performances of transgenic trees with altered lignification. *Nat Biotechnol* **20**: 607-612

282.���� Iglesias, V. A., Moscone, E. A., Papp, I., Neuhuber, F., Michalowski, S., Phelan, T., Spiker, S. *et al.* (1997). Molecular and cytogenetic analyses of stably and unstably expressed transgene loci in tobacco. *Plant Cell* **9**: 1251-1264

283.���� Matzke, A. J. & Matzke, M. A. (1998). Position effects and epigenetic silencing of plant transgenes. *Curr Opin Plant Biol* **1**: 142-148

284.���� Allen, G. C., Spiker, S. & Thompson, W. F. (2000). Use of matrix attachment regions (MARs) to minimize transgene silencing. *Plant Mol Biol* **43**: 361-376

285.���� Bender, J. & Fink, G. R. (1995). Epigenetic control of an endogenous gene family is revealed by a novel blue fluorescent mutant of Arabidopsis. *Cell* **83**: 725-734

286.���� Bender, J. (2004). DNA Methylation and Epigenetics. *Annu Rev Plant� Biol* **55**: 41-68

287.���� Gitlin, L., Karelsky, S. & Andino, R. (2002). Short interfering RNA confers intracellular antiviral immunity in human cells. *Nature* **418**: 430-434

288.���� Ebhardt, H. A., Thi, E. P., Wang, M. B. & Unrau, P. J. (2005). Extensive 3' modification of plant small RNAs is modulated by helper component-proteinase expression. *Proc Natl Acad Sci USA* **102**: 13398-13403

289.���� Vastenhouw, N. L. & Plasterk, R. (2004). RNAi protects the *Caenorhabditis elegans* germline against transposition. *Trends Genet* **20**: 314-319

290.���� Kakutani, T. (2002). Epi-Alleles in Plants: Inheritance of Epigenetic Information over Generations. *Plant Cell Physiol* **43**: 1106-1111

291.���� Lippman, Z., Gendrel, A. V., Black, M., Vaughn, M. W., Dedhia, N., McCombie, W. R., Lavine, K. *et al.* (2004). Role of transposable elements in heterochromatin and epigenetic control. *Nature* **430**: 471-476

292.���� Lippman, Z., May, B., Yordan, C., Singer, T. & Martienssen, R. (2004). Distinct Mechanisms Determine Transposon inheritance and Methylation via Small Interfering RNA and Histone Modification. *PLoS Biology* **1**: 420-428

293.���� Kamath, R. S., Martinez-Campos, M., Zipperlen, P., Fraser, A. G. & Ahringer, J. (2000). Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in Caenorhabditis elegans. *Genome Biol* **2**: 231-237

294.���� Bitko, V., Musiyenko, A., Shulyayeva, O. & S., B. (2005). Inhibition of respiratory viruses by nasally administered siRNA. *Nat Med* **11**: 50-55

295.���� Bitko, V. & Barik, S. (2001). Phenotypic silencing of cytoplasmic genes using sequence-specific double-stranded short interfering RNA and its application in the reverse genetics of wild type negative-strand RNA viruses. *BMC Microbiology* **1**: 34

296.���� Hutvagner, G., Simard, M. J., Mello, C. C. & Zamore, P. D. (2004). Sequence-specific inhibition of small RNA function. *PLoS Biology* **2**: e98

297.���� Song, J. J., Smith, S. K., Hannon, G. J. & Joshua-Tor, L. (2004). Crystal Structure of Argonaute and Its Implications for RISC Slicer Activity. *Science* **305**: 1434-1437

298.���� Yuan, Y. R., Pei, Y., Ma, J. B., Kuryavyi, V., Zhadina, M., Meister, G., Chen, H. Y. *et al.* (2005). Crystal Structure of A. aeolicus Argonaute, a Site-Specific DNA-Guided Endoribonuclease, Provides Insights into RISC-Mediated mRNA Cleavage. *Mol Cell* **19**: 405-419

299.���� Masse, E., Majdalani, N. & Gottesman, S. (2003). Regulatory roles for small RNAs in bacteria. *Curr Opin Microbiol* **6**: 120-124

300.���� Boutla, A., Kalantidis, K., Tavernarakis, N., Tsagris, M. & Tabler, M. (2002). Induction of RNA interference in Caenorhabditis elegans by RNAs derived from plants exhibiting post-transcriptional gene silencing. *Nucleic Acids Res* **30**: 1688-1694

301.���� Rosso, M. N., Dubrana, M. P., Cimbolini, N., Jaubert, S. & Abad, P. (2005). Application of RNA interference to root-knot nematode genes encoding esophageal gland proteins. *Mol Plant Microbe Interact* **18**: 615-620

302.���� Bakhetia, M., Charlton, W. L., Urwin, P. E., McPherson, M. J. & Atkinson, H. J. (2005). RNA interference and plant parasitic nematodes. *Trends in Plant Science* **10**: 362-367

303.���� Hewezi, T., Alibert, G. & Kallerhoff, J. (2005). Local infiltration of high- and low-molecular-weight RNA from silenced sunflower (Helianthus annuus L.) plants triggers post-transcriptional gene silencing in non-silenced plants. *Plant Biotechnol J* **3**: 81-89

304.���� Tenllado, F. & Diaz-Ruiz, J. R. (2001). Double-stranded RNA-mediated interference with plant virus infection. *J Virol* **75**: 12288-12297

305.���� Tenllado, F., Martinez-Garcia, B., Vargas, M. & Diaz-Ruiz, J. R. (2003). Crude extracts of bacterially expressed dsRNA can be used to protect plants against virus infections. *BMC Biotechnol* **3**

306.���� De Buck, S., Jacobs, A., Van Montagu, M. & Depicker, A. (1999). The DNA sequences of T-DNA junctions suggest that complex T-DNA loci are formed by a recombination process resembling T-DNA integration. *Plant J* **20**: 295-304

307.���� DeNeve, M., DeBuck, S., Jacobs, A., VanMontagu, M. & Depicker, A. (1997). T-DNA integration patterns in co-transformed plant cells suggest that T-DNA repeats originate from co-integration of separate T-DNAs. *Plant J* **11**: 15-29

308.���� Krizkova, L. & Hrouda, M. (1998). Direct repeats of T-DNA integrated in tobacco chromosome: characterization of junction regions. *Plant J* **16**: 673-680

309.���� Kumar, S. & Fladung, M. (2000). Transgene repeats in aspen: molecular characterisation suggests simultaneous integration of independent T-DNAs into receptive hotspots in the host genome. *Mol Gen Genet* **264**: 20-28

310.���� Eike, M. C., Mercy, I. S. & Aalen, R. B. (2005). Transgene silencing may be mediated by aberrant sense promoter sequence transcripts generated from cryptic promoters. *Cell Mol Life Sci* **62**

311.���� Nagaya, S., Kato, K., Ninomiya, Y., Horie, R., Sekine, M., Yoshida, K. & Shinmyo, A. (2005). Expression of randomly integrated single complete copy transgenes does not vary in Arabidopsis thaliana. *Plant and Cell Physiology* **46**: 438-444

312.���� De Buck, S., Windels, P., De Loose, M. & Depicker, A. (2004). Single-copy T-DNAs integrated at different positions in the Arabidopsis genome display uniform and comparable beta-glucuronidase accumulation levels. *Cell Mol Life Sci* **61**: 2632-2645

313.���� Aldwinckle, H. S., Borejsza-Wysocka, E. E., Malnoy, M., Brown, S. K., Norelli, J. L., Beer, S. V., Meng, X. *et al.* (2003). Development of fire blight resistant apple cultivars by genetic engineering. *Acta Horticulturae* **622**: 105-111

314.���� Rathore, K. S., Sunikumar, G., Puckhaber, L., Stipanovic, R. D., Nonjur, H. M., Hernandez, E. & Smith, C. W.(2003) in *10th IAPTC&B Congress* (ed. Vasil, I. K.) 417-420 (Kluwer Academic Publishers, Dordrecht, Netherlands, Orlando, Florida, USA).

315.���� Kawchuk, L. M. & Prufer, D. (1999). Molecular strategies for engineering resistance to potato viruses. *Can J Plant Pathol* **21**: 231-247

316.���� Vazquez Rovere, C., Asurmendi, S. & Hopp, H. E. (2001). Transgenic resistance in potato plants expressing potato leaf roll virus (PLRV) replicase gene sequences is RNA-mediated and suggests the involvement of post-transcriptional gene silencing. *Arch Virol* **146**: 1337-1353

317.���� Waterhouse, P. M., Smith, N. A. & Wang, M.-B. (1999). Virus resistance and gene silencing: killing the messenger. *Trends in Plant Science* **4**: 452-457

318.���� Neves-Borges, A. C., Collares, W. M., Pontes, J. A., Breyne, P., Farinelli, L. & Oliveira, D. E. d. (2001). Coat protein RNAs-mediated protection against Andean potato mottle virus in transgenic tobacco. *Plant Sci* **160**: 699-712

319.���� Scorza, R., Callahan, A., Levy, L., Damsteegt, V., Webb, K. & Ravelonandro, M. (2001). Post-transcriptional gene silencing in plum pox virus resistant transgenic European plum containing the plum pox potyvirus coat protein gene. *Transgenic Res* **10**: 201-209

320.���� Xu, J., Schubert, J. & Altpeter, F. (2001). Dissection of RNA-mediated ryegrass mosaic virus resistance in fertile transgenic perennial ryegrass (Lolium perenne L.). *Plant J* **26**: 265-274

321.���� Tennant, P., Fermin, G., Fitch, M. M., Manshardt, R. M., Slightom, J. L. & Gonsalves, D. (2001). Papaya ringspot virus resistance of transgenic Rainbow and SunUp is affected by gene dosage, plant development, and coat protein homology. *Eur J Plant Pathol* **107**: 645-653

322.���� Boogaart, T. v. d., Maule, A. J. & Davies, J. W. L., G. P. (2004). Sources of target specificity associated with the recovery against Pea seed-borne mosaic virus infection mediated by RNA silencing in pea. *Mol Plant Pathol* **5**: 37-43

323.���� Prins, M., Resende, R. D., Anker, C., Vanschepen, A., Dehaan, P. & Goldbach, R. (1996). Engineered RNA-mediated resistance to tomato spotted wilt virus is sequence-specific. *Mol Plant Microbe Interact* **9**: 416-418

324.���� He, X., Hall, M. B., Gallo-Meagher, M. & Smith, R. L. (2003). Improvement of forage quality by downregulation of maize O-methyltransferase. *Crop Science* **43**: 2240-2251

325.���� Allen, R. S., Millgate, A. G., Chitty, J. A., Thisleton, J., Miller, J. A. C., Fist, A. J., Gerlach, W. L. *et al.* (2004). RNAi-mediated replacement of morphine with the nonnarcotic alkaloid reticuline in opium poppy. *Nat Biotechnol* **22**: 1559-1566

326.���� Bradley, J. M., Rains, S. R., Manson, J. L. & Davies, K. M. (2000). Flower pattern stability in genetically modified lisianthus (Eustoma grandiflorum) under commercial growing conditions. *New Zealand Journal of Crop and Horticultural Science* **28**: 175-184

327.���� Marmiroli, N., Agrimonti, C., Visioli, G., Colauzzi, M., Guarda, G. & Zuppini, A. (2000). Silencing of G1-1 and A2-1 genes. Effects on general plant phenotype and on tuber dormancy in Solanum tuberosum L. *Potato Research* **43**: 313-323

328.���� Zeh, M., Casazza, A. P., Kreft, O., Roessner, U., Bieberich, K., Willmitzer, L., Hoefgen, R. *et al.* (2001). Antisense inhibition of threonine synthase leads to high methionine content in transgenic potato plants. *Plant Physiol* **127**: 792-802

329.���� Wolters, A. M. A. & Visser, R. G. F. (2000). Gene silencing in potato: allelic differences and effect of ploidy. *Plant Mol Biol* **43**: 377-386

330.���� Nakamura, R. & Matsuda, T. (1996). Rice allergenic protein and molecular-genetic approach for hypoallergenic rice. *Bioscience, Biotechnology and Biochemistry* **60**: 1215-1221

331.���� Tada, Y., Akagi, H., Fujimura, T. & Matsuda, T. (2003). Effect of an antisense sequence on rice allergen genes comprising a multigene family. *Breeding Science* **53**: 61-67

332.���� Lu, M. F., Liu, Q. Q., Chen, X. H., Yu, H. X., Wang, Z. Y. & Gu, M. H. (2003). Transformation of the antisense Waxy gene into the conventional indica rice varieties. *Journal of Yangzhou University, Agricultural and Life Sciences Edition* **24**: 32-36

333.���� Bhalla, P. L., Swoboda, I. & Singh, M. B. (1999). Antisense-mediated silencing of a gene encoding a major ryegrass pollen allergen. *Proc Natl Acad Sci USA* **96**: 11676-11680

334.���� Kimura, T., Otani, M., Noda, T., Ideta, O., Shimada, T. & Saito, A. (2001). Absence of amylose in sweet potato [Ipomoea batatas (L.) Lam.] following the introduction of granule-bound starch synthase I cDNA. *Plant Cell Rep* **20**: 663-666

335.���� Grossmann, K. & Schmulling, T. (1995). The effects of the herbicide quinclorac on shoot growth in tomato is alleviated by inhibitors of ethylene biosynthesis and by the presence of an antisense construct to the 1-aminocyclopropane-1-carboxylic acid (ACC) synthase gene in transgenic plants. *Plant Growth Regulation* **16**: 183-188

336.���� Escobar, M. A., Leslie, C. A., McGranahan, G. H. & Dandekar, A. M. (2002). Silencing crown gall disease in walnut (Juglans regia L.). *Plant Sci* **163**: 591-597

337.���� Boutla, A., Delidakis, C., Livadaras, I., Tsagris, M. & Tabler, M. (2001). Short 5 '-phosphorylated double-stranded RNAs induce RNA interference in Drosophila. *Curr Biol* **11**: 1776-1780

338.���� Boden, D., Pusch, O., Lee, F., Tucker, L. & Ramratnam, B. (2003). Human Immunodeficiency Virus Type 1 Escape from RNA Interference. *J Virol* **77**: 11531-11535

339.���� Kisielow, M., Kleiner, S., Nagasawa, M., Faisal, A. & Nagamine, Y. (2002). Isoform-specific knockdown and expression of adaptor protein ShcA using small interfering RNA. *Biochem J* **363**: 1-5

340.���� Wilda, M., Fuchs, U., Woessmann, W. & Borkhardt, A. (2002). Killing of leukemic cells with a BCR/ABL fusion gene by RNA interference (RNAi). *Oncogene* **21**: 5716-5724

341.���� Yu, J. Y., DeRuiter, S. L. & Turner, D. L. (2002). RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. *Proc Natl Acad Sci USA* **99**: 6047-6052

342.���� Amarzguioui, M., Holen, T., Babaie, E. & Prydz, H. (2003). Tolerance for mutations and chemical modifications in a siRNA. *Nucleic Acids Res* **31**: 589-595

343.���� Jones-Rhoades, M. W. & Bartel, D. P. (2004). Computational Identification of Plant MicroRNAs and Their Targets, Including a Stress-Induced miRNA. *Mol Cell* **14**: 787-799

344.���� Vazquez, F., Gasciolli, V., Crete, P. & Vaucheret, H. (2004). The Nuclear dsRNA Binding Protein HYL1 is Required for MicroRNA Accumulation and Plant Development, but not Posttranscriptional Transgene Silencing. *Curr Biol* **14**: 346-351

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Glossary of terms

- **Numbers**
  - 5' and 3' ends of DNA/RNA
- **A**
  - AGO1
  - AGO4
  - *Arabidopsis thaliana*
  - ARGONAUTE proteins
- **B**
  - Basepairs
  - Biosynthesis
- **C**
  - *Caenorhabditis elegans* (nematodes)
  - CG, CNG and CNN methylation
  - Chromatin
  - Chromodomain
  - Complementary sequences (DNA/RNA)
  - Co-suppression
- **D**
  - DCL1
  - DCL2
  - DCL3
  - DCL4
  - DDM1
  - Dicer
  - Domain
  - DRM methyltransferase
  - *Drosophila melanogaster* (fruitfly)
- **E**
  - EGS1/EGS2
  - Endogene
  - Endogenous siRNAs
  - Epigenetic
  - Expression
- **G**
  - Genome
- **H**
  - Hairpin RNA
  - HEN1
  - Heterochromatin
  - Heterochromatinisation
  - Homology
  - HYL1

- **I**
    - Imprinting
    - Inverted repeat sequences
    - *In-vitro* and *in-vivo*
- **M**
    - MET1
    - Methylation
    - Methyltransferase
    - Microarray
    - Micro RNA (miRNA)
    - Mismatches and wobble basepairs
- **N**
    - Nuclease
- **O**
    - Off-target
- **P**
    - Position effect variegation (PEV)
    - Post-transcriptional
    - Primer
    - Promoter
- **R**
    - R2D2
    - RDR1
    - RDR2
    - RDR6
    - Reporter genes
    - Rgs-CaM
    - Ribozyme
    - RISC (RNA-induced silencing complex)
    - RITS (RNA-induced initiation of transcriptional gene silencing complex)
    - RNA-dependent RNA polymerase
    - RNAi
- **S**
    - SDE1
    - Sense and antisense sequences (DNA/RNA)
    - Sequence
    - SGS2
    - SGS3
    - Short hairpin RNA (shRNA)
    - Small interfering RNA (siRNA)
    - Size classes of siRNA
    - Suppressors of gene silenicng
- **T**
    - Trans-acting siRNA
    - Transcription
    - Transcriptome

  - Transitivity
  - Transposons
- U
  - Uridylation
- **V**
  - Viroid
- **W**
  - Wobble

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Images

The images, or series of images, in this section show the main pathways of RNA silencing and illustrate some of the concepts introduced in the report.

Select the topic below:

- **General**
  - General overview (clickable map of triggers and effects of silencing pathways)
  - Introduction to transcripton and translation (including effects of silencing pathways)
  - Types of repeats (RNA)
  - siRNA
- **Post-trancriptional RNA silencing**
  - triggered by single-stranded RNA
    - Overview
    - Step by step
  - triggered by a virus
    - Overview
    - Step by step
  - Spreading of RNA silencing
    - Spreading of silencing through a plant (systemic silencing)
    - Spreading of target region (transitivity**)**
- **Endogenous small RNA pathways**
  - miRNA pathways
    - Overview
    - Step by step
  - Other endogenous small RNAs (methylation/chromatin-modification)
    - Overview
- **Transgene induced-silencing strategies**
      - post-transcriptional vs. transcriptional silencing
- **Hypothetical hazards associated with RNA silencing in GM crops**
  - Off-target effects
  - Instability of silencing
  - Suppression of RNA silencing by plant viruses
  - Escape of viruses from silencing-based resistance
  - Saturation of the silencing machinery
  - Horizontal transfer of RNA silencing

Online survey

To find out more about unpublished experiences with silencing-based transgenic plants, we conducted a survey among research groups working on RNA silencing in plants. A total of 195 scientists who had published peer-reviewed articles relevant to the subject were invited to fill out an online questionnaire, which was designed and published using the Perseus SurveySolutions� Express tool (http://express.perseus.com). We received 38 completed questionnaires. One person specified that he/she was not working on plants but had been involved in off-target studies in other organisms. Nine participants were involved in the development of commercial silencing-based GM crops but only two specified to be working in industry, the other 36 have positions in academia, 19 of those are research group leaders.

The majority of participants (35 of 38) uses RNA silencing as a tool to study functions of other genes, 19 study the mechanism of RNA silencing itself and 16 specified to use RNA silencing to produce GM crops (more than one option was allowed).

As discussed in section 5 of the report, many researchers still use single-stranded sense or antisense RNA as a trigger of RNA silencing. These strategies, known as co-suppression or antisense suppression, are used by 22 of 38 participants. Long double-stranded (hairpin) RNA and short hairpin (sh)RNA constructs are used by 25 and 7 participants respectively, and 15 have used virus induced gene silencing (more than one option allowed). This shows that the more efficient double-stranded triggers are gaining popularity and that an increase in the use of shRNAs in published studies can be expected in the near future. Given the potential of these short triggers in avoiding off-target effects, this is an important development.

We asked how researchers ensured optimal efficiency and minimal off-target effects in the design of their silencing triggers. The BLAST algorithm is used by 21 of 38 participants and 22 participants specifically look for potential target sites within messenger RNAs of known family members of the gene of interest. Web-based tools for siRNA design and other non-specified methods are used by 7 participants each (more than one option was allowed). None of these methods for ensuring efficient and specific silencing are used by 6 participants. The majority therefore uses computational tools in the design process of silencing triggers.

We wanted to know how many silencing-based transgenic plant lines the participants had worked with so far. Thirty-five participants answered this question and the majority of 21 participants specified to have worked with 1-20 lines of such plants, while 12 had worked with more than 50 lines. In addition, non-stable (transient) silencing constructs were used by 25 participants, 17 of which had used between 1 and 20 different constructs.

Although most researchers seem to use computational tools to ensure specific targeting by their silencing-trigger constructs, only 8 participants specified to routinely check for off-target effects in silenced plants. Another 12 do check sometimes but not on a routine basis. Of those 20 participants who check off-target effects, 13 examine transcript levels only whereas 4 check the translation of putative non-target genes as well. A further 3 specified to sometimes, but not routinely, check for translational repression. Translational repression is often difficult to analyse because antibodies for the detection of specific proteins are not always available. Translational repression is not well examined in plants but it has a strong off-target potential because it is induced by rather weak siRNA-target interactions that may have a substantial number of mismatches.

We asked the 20 participants who check for off-target silencing (occasionally or routinely) to give an estimate of the frequency at which this occurs with the various techniques they use.� For each of the techniques, participants were asked whether they have used the technique and are able to estimate what percentage of plants they found to exhibit any off-target silencing, choices were: 0%, less than 1%, between 1 and 10%, between 50 and 90% or more than 90%.� One participant specified to have found off-target silencing in more than 90% of plants when using co-suppression or antisense suppression. All other participants had observed significantly fewer off-target effects. The four other participants who specified their estimates for off-target silencing with co-suppression or antisense suppression found off-target silencing in less than 1% (one participant) or none of the plants (3 participants). Using long double-stranded RNA as trigger, three participants estimated to have seen off-target effects in 1 to 10% of their plants, one participant in less than 1%, and 7 found no off-target effects in the plants they had worked with at all. All three participants who gave an estimate of the frequency of off-target effects using virus-induced transient gene silencing (VIGS) specified to have observed no off-target effects in their experiments. Two participants gave an estimate for shRNA constructs, of which one specified to have observed no off-target effects and one found off-target silencing in 1 to 10% of the plants. One participant had analysed off-target effects when using siRNAs in plants and found none. Two participants used other methods than the ones represented on the questionnaire to obtain silenced plants and both estimated to have seen off-target effects in less than 1% of plants. Two participants did not specify the silencing technique used but specified to have found no off-target effects in silenced plants. In summary, no more than 10% of silenced plant lines were estimated to exhibit off-target effects by all but one of the participants. However, we do not know how exactly potential off-target effects analyses were carried out in these cases. Furthermore, some of the observed off-target effect may in fact be secondary effects as a consequence of silencing the target gene.

Two of 38 participants specified to have been involved in large-scale studies of off-target effects. One of them found no non-target messenger RNA to be affected by the presence of the silencing trigger and the other one found less than 1% of messenger RNAs to be affected.

Switching from post-transcriptional to transcriptional silencing in transgenic plants is a possibility that has not received a lot of attention in published studies so far. We asked the participants whether they determine the mode of silencing (post-transcriptional or transcriptional) in their experiments. Thirteen of 38 participants normally examine the mode of silencing in their transgenic plants and a further 5 specified to do this sometimes. Asked for the percentage of plants that exhibit transcriptional silencing but were designed for post-transcriptional silencing, 1 participant specified between 50 and 90%, 6 participants between 1 and 10%, 4 participants observed this in less than 1% and 8 participants in none of the transformed plants. Therefore, unintentional triggering of transcriptional silencing when using post-transcriptional silencing strategies is usually observed in no more than 10% of plant lines by most of the participants.

We also asked participants whether they examine the stability of silencing over a number of generations. Thirty of 38 participants specified to do this, 5 of which normally check more than 4 generations, 15 participants check 3-4 generations and 10 examine 1-2 generations. The remaining 8 participants do not check the stability of the silenced state in their transgenic plants. Of those 30 participants who do check the stability, 13 never found instability, 8 found loss of silencing to occur very rarely, 8 found an occasional loss of silencing and one specified loss of silencing to occur frequently. The 17 participants who specified to find loss of silencing at least in some cases were asked whether they check the methylation status of the silencing trigger transgene in the cases where silencing has been lost. Fifteen of these 17 never analysed this and the two that did specified to find increased methylation to be correlated with a loss of silencing in at least some of the cases. Therefore the majority of participants would normally analyse the stability of silencing in transgenic plant lines over at least 1-2 generations and although loss of silencing is observed, only one participant would classify this as a frequent event.

Finally, all participants were given the opportunity of adding further comments. One participant raised the question of how to define off-target effects as discussed in the report. One participant wrote �We have had lines in which silencing was enhanced between T2 and T3 generations.� This pattern was not described in any of the publications reviewed in this report and may be a rare case which, in general, should not cause a problem in the transgenic plant, its environment or the consumer.

Another participant commented: �As I observed, RNAi technology is not so efficient or stable as some papers have declared. However, gene silencing efficiency in some of the transformants are satisfactory, and these transformants selected are valuable. They can be used for further investigations.�

One comment highlights a particular problem for comparative literature studies. This participant wrote �[...] I also worked with transgenic lettuce during my PhD, but from the third generation on plants lost resistance due to methylation and the work was never published.� The generation of virus resistant plants using RNA silencing technologies is not novel anymore and therefore difficult to publish in peer reviewed high-impact journals. In addition, the experiment was unsuccessful in this case, as the resistance turned out to be unstable. From a risk assessment point of view, however, results like these are interesting because we need to know how frequently researchers find an instable silencing phenotype. For a comprehensive analysis it would be ideal to have a large number of similar experiments, failed or successful from the experimenter�s point of view, published in the scientific literature. To complement this limitation of the peer-reviewed publishing process for scientific data, surveys like the one presented here might help to get a more comprehensive picture of the unpublished expertise of researchers.

Sequence

The term "sequence" either refers to the sequence of nucleobases in DNA or RNA polymers (chains) or the sequence of amino acids that make up a protein. The nucleobase sequence of genomic DNA is transcribed into messenger RNA, which is translated into protein.

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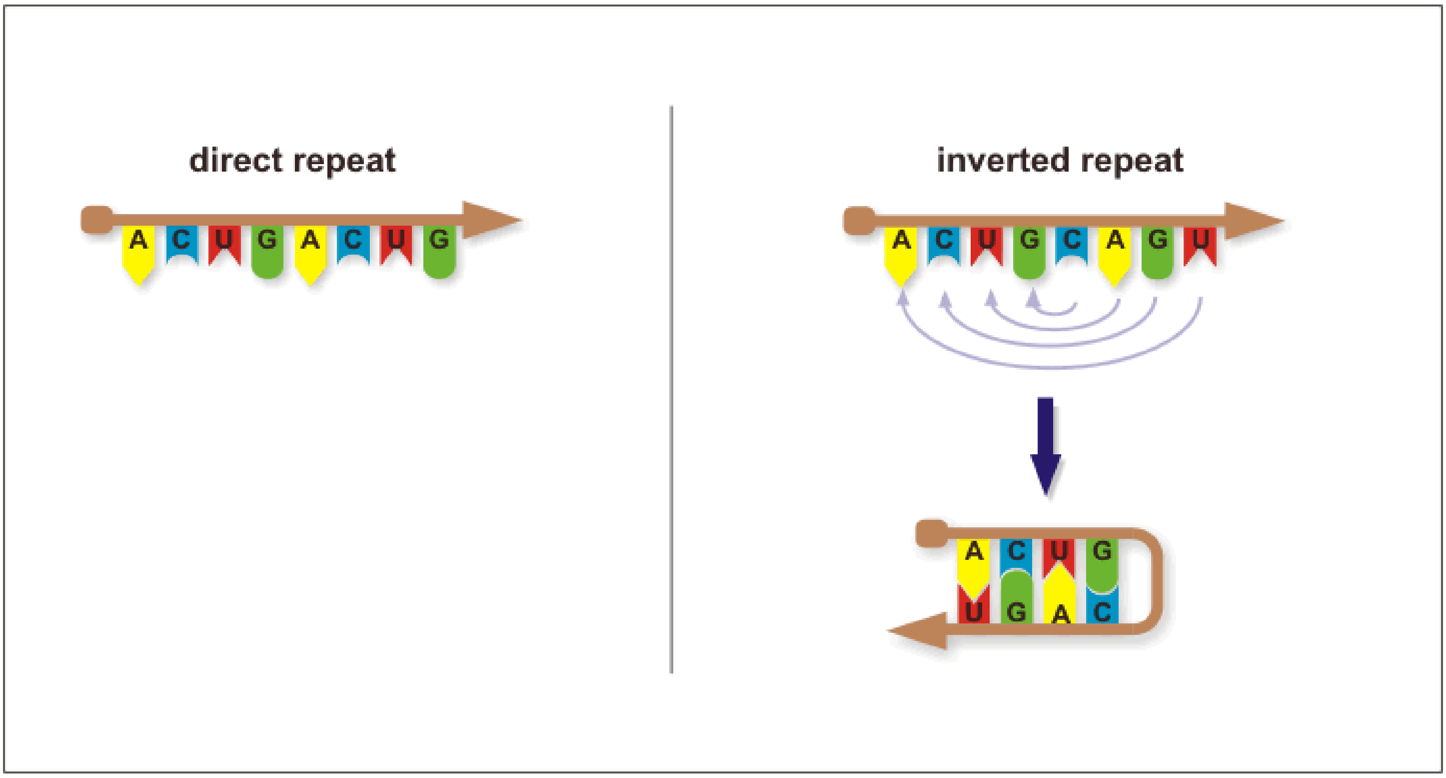
Direct and inverted repeats



DNA or RNA often contains regions of repeated sequences.

Two single-stranded RNAs are shown here. The molecule on the left contains a direct repeat of the nucleobase sequence (A)denine - (C)ytosine - (U)racil - (G)uanine. The inverted repeat shown on the right is obtained by repeating the sequence in reverse order (ACUG > GUCA) and then forming the complement (GUCA > CAGU). Because of the complementarity of one part of the RNA strand to another part, an intramolecular interaction is possible that results in a double-stranded "hairpin" structure. An RNA with direct repeats does not form this structure.

Since double-stranded RNA is a very potent trigger of RNA silencing, inverted repeat sequences can be used to efficiently induce silencing in plants and other organisms. These can be long sequences that encompass the entire transcribed region of the target gene, or they can be as short as an siRNA. The latter are referred to as short hairpin (sh)RNAs.

Mismatches and wobble basepairs

Nucleobases in DNA and RNA molecules are complementary to each other, i.e. adenine normally forms basepairs with thymine (in DNA) or uracil (in RNA) and guanine pairs with cytosine. A mismatch is a combination of nucleobases on the two opposing strands that can not form a basepair. However, there are some less-frequent combinations of nucleobases that can form hydrogen bonds. In RNA, guanine often pairs with uracil, which is termed a �wobble� basepair.

Wobble basepairs, unlike complete mismatches, do not disturb the spatial geometry of the double helix. In the siRNA-target interaction, wobble basepairs as well as the A-C mismatch can be tolerated well [1] .

Literature

1.�������� Du, Q., Thonberg, H., Wang, J., Wahlestedt, C. & Liang, Z. (2005). A systematic analysis of the silencing effects of an active siRNA at all single-nucleotide mismatched target sites. *Nucleic Acids Res* **33**: 1671-1677

Small interfering (si)RNA

A ground breaking study by Hamilton and Baulcombe in 1999 identified a novel class of signalling RNA molecules that are indicative of silencing processes [1] . These were named small (or short) interfering (si)RNAs and are now known to be central to all RNA silencing pathways. SiRNAs are generated from double-stranded RNAs by a Dicer enzyme [2] . However, siRNAs are not just the end-product of RNA silencing - they mediate the sequence specificity of RNA silencing by binding to target RNA and inducing its destruction [3,4] (see Figure).
SiRNAs are double-stranded because they are excised from longer double-stranded RNAs. The siRNA duplex is furthermore characterised by overhangs of 2 nucleotides at the 3� ends of both strands, which are due to the staggered cuts that the Dicer enzymes introduce in the trigger RNA [2] (Figure). Therefore, only 19 nucleotides of a 21 nucleotide siRNA are actually base-paired with the complementary strand [4] . The 5� ends of both strands of the siRNA duplex are phosphorylated and the 3� ends are hydroxylated [4] .
Although siRNAs are double-stranded, the two strands are not equally loaded into the silencing effector complex (RISC) to guide the degradation of target RNA. Selecting the guide strand is not a random process. Instead, a protein probes the stability of the two ends of the double-stranded structure [5] . The differential stability of the ends is due to the fact that adenine-uracil basepairs are weaker than cytosine-guanine basepairs. The weaker end, i.e. the one with more A-U basepairs, is then presented to other components of the silencing machinery that separate the strands and incorporate the guide strand into RISC [6,7] .
The development of protocols for the sequencing of siRNAs from living cells has revealed the existence of siRNAs that are not derived from transgenes or viruses. SiRNAs that are derived from the organism�s own genes are now referred to as endogenous siRNAs [8,9,10,11] . Some of these are derived from transposable elements and play an important role in protecting the genome from the mutagenic action of these mobile genetic elements [12] . Another group of endogenous siRNAs regulates the expression of other transcripts in a micro (mi)RNA-like manner. These are called trans-acting (ta-) siRNAs.
Initially, siRNAs were thought to be of uniform length of about 25 nucleotides [1] . Later, different size classes ranging from 21 to 27 nucleotides were found, which are often linked to specific silencing phenomena [13] . The two major size classes in plants are �short� (21-22 nucleotides) and �long �(24-26 nucleotides) siRNAs. While transgene-induced silencing gives rise to siRNAs of both classes, silencing of transposons (endogenous targets) is associated with long siRNAs only [13] . More evidence for distinct roles of the two size classes came from analyses using viral suppressors of RNA silencing. Different suppressors differentially affecteded the accumulation of the two size classes of siRNAs from a double-stranded transgene RNA [13] . The observation that those suppressors that affected systemic movement of RNA silencing through the plant also inhibit the accumulation of long siRNAs led to the hypothesis that long siRNAs are the systemic RNA silencing signal that travels through the phloem to trigger sequence specific silencing throughout the plant [13,14] . However, this hypothesis has not been confirmed yet and other candidates for the mobile silencing signal are also discussed [14] . Long siRNAs were furthermore found to be required for RNA-directed DNA methylation [13] . Short siRNAs, in contrast, are sufficient for silencing of a transgene in a cell in which the silencing trigger RNA is present [13] . In addition, short siRNAs have been shown to mediate the local cell-to-cell spreading of RNA silencing [15] .
Normally, siRNAs silence target RNAs by introducing a cleavage in the target RNA in the centre of their binding site [4] . The cleavage products are subsequently degraded in a process that might involve different protein components for the two fragments [16] .
Most animal miRNAs seem to interfere with the translation of their target messenger RNAs rather than causing their degradation. By introducing central mismatches, siRNAs can also be forced to mimic this animal miRNA-like mode of action [17] .
SiRNAs are now routinely synthesised artificially as a research tool to mediate RNA silencing *in-vitro* and *in-vivo* [4,18] . Furthermore, synthetic siRNAs are currently being developed into a novel type of drug to fight infectious diseases and genetic disorders [19,20,21] .

Literature

1.��������� Hamilton, A. J. & Baulcombe, D. C. (1999). A species of small antisense RNA in post-transcriptional gene silencing in plants. *Science* **286**: 950-952

2.��������� Bernstein, E., Caudy, A. A., Hammond, S. M. & Hannon, G. J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409**: 363-366

3.��������� Zamore, P. D., Tuschl, T., Sharp, P. A. & Bartel, D. P. (2000). RNAi: Double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* **101**: 25-33

4.��������� Elbashir, S. M., Lendeckel, W. & Tuschl, T. (2001). RNA interference is mediated by 21-and 22-nucleotide RNAs. *Genes Dev* **15**: 188-200

5.��������� Tomari, Y., Matrange, C., Haley, B., Martinez, N. & Zamore, P. D. (2004). A Protein Sensor for siRNA Asymmetry. *Science* **306**: 1377-1380

6.��������� Schwarz, D. S., Hutvagner, G., Du, T., Xu, Z., Aronin, N. & Zamore, P. D. (2003). Asymmetry in the Assembly of the RNAi Enzyme Complex. *Cell* **115**: 199-208

7.��������� Khvorova, A., Reynolds, A. & Jayasena, S. D. (2003). Functional siRNAs and miRNAs Exhibit Strand Bias. *Cell* **115**: 209-216

8.��������� Llave, C., Kasschau, K. D., Rector, M. A. & Carrington, J. C. (2002). Endogenous and silencing-associated small RNAs in plants. *Plant Cell* **14**: 1605-1619

9.��������� Sunkar, R. & Zhu, J.-K. (2004). Novel and Stress-Regulated MicroRNAs and Other Small RNAs from Arabidopsis. *Plant Cell* **16**: 2001-2019

10.������� Vazquez, F., Vaucheret, H., Rajagopalan, R., Lepers, C., Gasciolli, V., Mallory, A. C., Hilbert, J.-L. *et al.* (2004). Endogenous trans-acting siRNAs Regulate the Accumulation of Arabidopsis mRNAs. *Mol Cell* **16**: 69-79

11.������� Gustafson, A. M., Allen, E., Givan, S., Smith, D., Carrington, J. C. & Kasschau, K. D. (2005). ASRP: the Arabidopsis Small RNA Project Database. *Nucleic Acids Res* **33**: D637-D640

12.������� Waterhouse, P. M., Wang, M. B. & Lough, T. (2001). Gene silencing as an adaptive defence against viruses. *Nature* **411**: 834-842

13.������� Hamilton, A. J., Voinnet, O., Chappell, L. & Baulcombe, D. C. (2002). Two classes of short interfering RNA in RNA silencing. *EMBO J* **21**: 4671-4679

14.������� Mlotshwa, S., Voinnet, O., Mette, M. F., Matzke, M., Vaucheret, H., Ding, S. W., Pruss, G. *et al.* (2002). RNA silencing and the mobile silencing signal. *Plant Cell* **14 Suppl**: S289-301

15.������� Himber, C., Dunoyer, P., Moissiard, G., Ritzenthaler, C. & Voinnet, O. (2003). Transitivity-dependent and -independent cell-to-cell movement of RNA silencing. *EMBO J* **22**: 4523-4533

16.������� Orban, T. I. & Izaurralde, E. (2005). Decay of mRNAs targeted by RISC requires XRN1, the Ski complex, and the exosome. *RNA* **11**: 459-469

17.������� Doench, J. G., Petersen, C. P. & Sharp, P. A. (2003). siRNAs can function as miRNAs. *Genes Dev* **17**: 438-442

18.������� Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. & Tuschl, T. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**: 494-498

19.������� Barik, S. (2005). Silence of the transcripts: RNA interference in medicine. *J Mol Med* **83**: 764-773

20.������� Barik, S. (2004). Development of gene-specific double-stranded RNA drugs. *Ann Med* **36**: 540-

551

21.������ Whelan, J. (2005). First clinical data on RNAi. *Drug Discovery Today* **10**: 1014-1015

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Micro (mi)RNA

Micro (mi)RNAs are small RNAs (21-24 nucleotides long) that regulate gene expression in plants and animals but are absent in fungi. They have an important role in growth and development of the organism. Like siRNAs, miRNAs feed into the post-transcriptional gene silencing pathway, leading either to degradation of the target messenger RNA or to translational repression [1] . The main difference between miRNAs and siRNAs is that miRNAs are encoded by a distinct class of genes in the organism�s own genome. The transcripts from these genes do not encode proteins. Instead, the transcribed RNA is partially self-complementary, which enables it to fold into a characteristic structure that includes imperfect double-stranded regions from which the miRNAs are excised [1] . The excision of the mature miRNA is a multi-step reaction that involves trimming the initial precursor several times. In plants, this reaction, at least in part, is performed by the Dicer enzyme DCL1 [2] . Only one strand of the initially double-stranded miRNA is incorporated selectively in the silencing effector complex RISC to guide it to its target messenger RNA [3,4] . In the fruitfly *Drosophila melanogaster*, this selection is carried out by the R2D2 protein. This protein probes the binding strength of the two ends of the double-stranded miRNA. The weaker end is presented to other proteins which separate the two strands and hand over the functional (guiding) strand to RISC [5] .

The proteins HYL1 [6,7] and HEN1 [8,9] are also required for miRNA accumulation and thus normal development in Arabidopsis. HEN1 modifies miRNAs by adding methyl groups to the ribose backbone [10] . The methylation might increase the stability of the mature miRNA and prevent it from serving as a primer for RNA-polymerases.

MicroRNAs are produced in the nucleus but act in the cytoplasm. In animals, the protein Exportin5 acts as a shuttle to transport miRNAs from the nucleus into the cytoplasm [11,12,13] . This role might be carried out in plants by the Exportin5-homologoue HASTY [14] .

Apart from their biosynthesis, miRNAs are characterised by their conservation between species. Most families of *Arabidopsis* miRNAs have obvious homologues in rice and other plants [15,16,17,18] . However, plant miRNA only show homology to other plant miRNAs and animal miRNAs to animal miRNAs [19] , indicating that this mechanism of gene-regulation has evolved separately in plants and animals.

In plants, miRNAs initiate cleavage of the target messenger RNA which is then degraded [20,21,22,23] . In contrast, the default mode of action of animal miRNAs is thought to be translational repression of the messenger RNA, i.e. the target is not degraded but prevented from being translated into protein [24,25,26,27,28] . In mammalian cells, messenger RNAs that are subject to translational repression are �rounded up� in compartments called P-bodies [29] , where they might be degraded [30] .

The differential effect of animal and plant miRNAs on target messenger RNAs is reflected in the degree of similarity between miRNA and target: animal miRNAs generally exhibit more mismatches to their target than plant miRNAs, which seems to prevent the cleavage reaction [26,31,32,33] . However, there are plant miRNAs that cause translational arrest rather than target degradation and animal RNAs that induce degradation of the messenger RNA [34,35,36,37,38] . Furthermore, a recent report showed that at least two nematode miRNAs that have originally be classified as translational repressors actually cause degradation of the target messenger RNA [34] .

Another difference between animal and plant miRNAs is the binding site within the messenger RNA: animal miRNAs often target multiple sites within the 3� untranslated region of the messenger RNA [24,25,39,40,41,42,43,44,45,46] while plant miRNAs bind to a single site anywhere within the target [47] . It has been reported that translational repression is far less efficient than target degradation, which may explain why multiple target sites in animal messenger RNAs are necessary [31] . However, in one study a single binding site for an imperfectly matched siRNA�was shown to be sufficient to induce translational repression [33] .

Although many small RNAs, including miRNAs, from plants have been cloned and sequenced (see http://asrp.cgrb.oregonstate.edu/db/), the number of validated targets of miRNAs is still small. Identifying targets is easier in plants than in animals because plant miRNAs generally exhibit a high degree of complementarity to their target sites, which facilitates computational target identification [47,48] . Animal miRNAs, in contrast, tend to bind their targets rather loosely with many mismatches. Given the small size of miRNAs, this greatly complicates computer-aided target prediction [49] .

To refine the search for miRNA targets it is important to test and update the known rules for miRNA-target interaction. The most recent update of miRNA-target recognition rules comes from a large-scale study of genome-wide miRNA-mediated gene regulation [50] .

The impact of a miRNA on gene expression can be complex. In animals, miRNAs can shift the entire transcription

profile of a cell, which indicates a pivotal role for miRNAs in establishing and maintaining tissue identity [38]. In plants, many miRNA targets encode transcription factors, which in turn regulate specific subsets of genes. In addition, the biosynthesis of another class of regulative small RNAs, so called trans-acting siRNAs, has recently been shown to depend on miRNAs [51]. Thus miRNAs can be �regulators of regulators� [52].
Bearing in mind this complexity of miRNA-mediated gene regulation, it is easy to imagine that any disruption of this mechanism must have severe consequences. Viruses can interfere with silencing pathways including miRNA-mediated silencing by encoding proteins that suppress RNA silencing. It has been suggested that many symptoms of viral diseases in plants are caused by the disruption of miRNA-mediated gene regulation by silencing suppressor proteins [53,54,55,56,57,58]. However, viral suppressors are highly diverse and not all of them affect miRNA-mediated silencing [56].
Two new layers of complexity have recently been added to our knowledge on the relationship between viruses and miRNAs. One of them is that hosts can encode miRNA genes which target specific viruses [59]. This constitutes a novel form of heritable sequence-specific immunity against viruses and, as expected, viruses seem to have evolved proteins to suppress this mechanism [59]. The other one is that viruses themselves can encode miRNAs, which can target host genes and viral genes, which might contribute to regulating the viral infection cycle [60].

Literature

1.��������� Dugas, D. V. & Bartel, B. (2004). MicroRNA regulation of gene expression in plants. *Curr Opin Plant Biol* **7**: 512-520

2.��������� Kurihara, Y. & Watanabe, Y. (2004). Arabidopsis micro-RNA biogenesis through Dicer-like 1 protein functions. *Proc Natl Acad Sci USA* **101**: 12753-12758

3.��������� Khvorova, A., Reynolds, A. & Jayasena, S. D. (2003). Functional siRNAs and miRNAs Exhibit Strand Bias. *Cell* **115**: 209-216

4.��������� Schwarz, D. S., Hutvagner, G., Du, T., Xu, Z., Aronin, N. & Zamore, P. D. (2003). Asymmetry in the Assembly of the RNAi Enzyme Complex. *Cell* **115**: 199-208

5.��������� Tomari, Y., Matrange, C., Haley, B., Martinez, N. & Zamore, P. D. (2004). A Protein Sensor for siRNA Asymmetry. *Science* **306**: 1377-1380

6.��������� Han, M.-H., Goud, S., Song, L. & Fedoroff, N. (2004). The *Arabidopsis* double-stranded RNA-binding protein HYL1 plays a role in microRNA-mediated gene regulation. *Proc Natl Acad Sci USA* **101**: 1093-1098

7.��������� Vazquez, F., Gasciolli, V., Crete, P. & Vaucheret, H. (2004). The Nuclear dsRNA Binding Protein HYL1 is Required for MicroRNA Accumulation and Plant Development, but not Posttranscriptional Transgene Silencing. *Curr Biol* **14**: 346-351

8.��������� Park, W., Li, J., Song, R., Messing, J. & Chen, X. (2002). CARPEL FACTORY. a Dicer homolog, and HEN1, a novel protein, act in microRNA metabolism in *Arabidopsis thaliana*. *Curr Biol* **12**: 1484-1495

9.��������� Boutet, S., Vazquez, F., Liu, J., Beclin, C., Morel, J.-B., Crete, P., Chen, X. *et al.* (2003). *Arabidopsis HEN1*: A Genetic Link between Endogenous miRNA Controlling Development and siRNA Controlling Transgene Silencing and Virus Resistance. *Curr Biol* **13**: 843-848

10.������� Yu, B., Yang, Z., Li, J., Minakhina, S., Yang, M., Padgett, R. W., Steward, R. *et al.* (2005). Methylation as a Crucial Step in Plant microRNA Biogenesis. *Science* **307**: 932-935

11.������� Yi, R., Qin, Y., Macara, I., G. & Cullen, B. R. (2003). Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes Dev* **17**: 3011-3016

12.������ Bohnsack, M. T., Czaplinksi, K. & G�rlich, D. (2004). Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. *RNA* **10**: 185-191

13.������ Lund, E., Guttinger, S., Calado, A., Dahlberg, J. E. & Kutay, U. (2004). Nuclear Export of MicroRNA Precursors. *Science* **303**: 95-98

14.������ Bollmann, K. M., Aukerman, M. J., Park, M. Y., Hunter, C., Berardini, T. Z. & Poethig, R. S. (2003). HASTY, the *Arabidopsis* ortholog of exportin 5/MSN5, regultes phase change and morphogenesis. *Development* **130**: 1493-1504

15.������ Bartel, B. & Bartel, D. P. (2003). MicroRNAs: At the Root of Plant Development? *Plant Physiol* **132**: 709-717

16.������ Floyd, S. K. & Bowman, J. L. (2004). Gene regulation: Ancient microRNA target sequences in plants. *Nature* **428**: 485-486

17.������ Axtell, M. J. & Bartel, D. P. (2005). Antiquity of MicroRNAs and Their Targets in Land Plants. *Plant Cell* **17**: 1658-1673

18.������ Reinhart, B. J., Weinstein, E. G., Rhoades, M., Bartel, B. & Bartel, D. P. (2002). MicroRNAs in plants. *Genes Dev* **16**: 1616-1626

19.������ Pasquinelli, A. E., Reinhart, B. J., Slack, F., Martindale, M. Q., Kuroda, M. I., Maller, B., Hayward, D. C. *et al.* (2000). Conservation of the sequence and temporal expression of *let-7* heterochronic regulatory RNA. *Nature* **408**: 86-89

20.������ Llave, C., Xie, Z., Kasschau, K. D. & Carrington, J. C. (2002). Cleavage of *Scarecrow-like* mRNA targets directed by a class of *Arabidopsis* miRNA. *Science* **297**: 2053-2056

21.������ Kasschau, K. D., Xie, Z., Allen, E., Llave, C., Chapman, E. J., Krizan, K. A. & Carrington, J. C. (2003). P1/HC-Pro, a Viral Suppressor of RNA Silencing, Interferes with Arabidopsis Development and miRNA Function. *Dev Cell* **4**: 205-217

22.������ Tang, G., Reinhart, B. J., Bartel, D. P. & Zamore, P. D. (2003). A biochemical framework for RNA silencing in plants. *Genes Dev* **17**: 49-63

23.������ Orban, T. I. & Izaurralde, E. (2005). Decay of mRNAs targeted by RISC requires XRN1, the Ski complex, and the exosome. *RNA* **11**: 459-469

24.������ Lee, R. C., Feinbaum, R. L. & Ambros, V. (1993). The C-Elegans Heterochronic Gene Lin-4 Encodes Small Rnas with Antisense Complementarity to Lin-14. *Cell* **75**: 843-854

25.������ Reinhart, B. J., Slack, F. J., Basson, M., Pasquinelli, A. E., Bettinger, J. C., Rougvie, A. E., Horvitz, H. R. *et al.* (2000). The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. *Nature* **403**: 901-906

26.������ Hutvagner, G. & Zamore, P. D. (2002). A microRNA in a multiple-turnover RNAi enzyme complex. *Science* **297**: 2056-2060

27.������ Pasquinelli, A. F. & Ruvkun, G. (2002). Control of developmental timing by microRNAs and their targets. *Annu Rev Cell Dev Biol* **18**: 495-513

28.������ Ambros, V. (2003). MicroRNA pathways in flies and worms: growth, death, fat, stress, and timing. *Cell* **113**: 673-676

29.������ Sen, G. L. & Blau, H. M. (2005). Argonaute 2/RISC resides in sites of mammalian mRNA decay known as cytoplasmic bodies. *Nat Cell Biol* **7**: 633-636

30.������ Liu, J., Valencia-Sanchez, M. A., Hannon, G. J. & Parker, R. (2005). MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies. *Nat Cell Biol* **7**: 719-723

31.������ Doench, J. G., Petersen, C. P. & Sharp, P. A. (2003). siRNAs can function as miRNAs. *Genes Dev* **17**: 438-442

32.������ Zeng, Y., Yi, R. & Cullen, B. R. (2003). MicroRNAs and smalll interfering RNAs can inhibit mRNA expression by similar mechanisms. *Proc Natl Acad Sci USA* **100**: 9779-9784

33.������ Saxena, S., Jonsson, Z. O. & Dutta, A. (2003). Small RNAs with imperfect match to endogenous mRNA repress translation. Implications for off-target activity of small inhibitory RNA in mammalian cells. *J Biol Chem* **27**: 44312-44319

34.������ Bagga, S., Bracht, J., Hunter, S., Massirer, K., Holtz, J., Eachus, R. & Pasquinelli, A. E. (2005). Regulation by let-7 and lin-4 miRNAs Results in Target mRNA Degradation. *Cell* **122**

35.������ Yekta, S., Shih, I. H. & Bartel, D. P. (2004). MicroRNA-directed cleavage of HOXB8 mRNA. *Science* **304**: 594-596

36.������ Aukerman, M. J. & Sakai, H. (2003). Regulation of Flowering Time and Floral Organ Identity by a MicroRNA and its *APETALA2*-Like Target Genes. *Plant Cell* **15**: 2730-2741

37.������ Chen, X. M. (2004). A microRNA as a translational repressor of APETALA2 in Arabidopsis flower development. *Science* **303**: 2022-2025

38.������ Lim, L. P., Lau, N. C., Garrett-Engele, P., Grimson, A., Schelter, J. M., Castle, J., Bartel, D. P. *et al.* (2005). Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature* **433**: 769-773

39.������ Bartel, D. P. (2004). MicroRNAs: Genomics, Biogenesis, Mechanism, and Function. *Cell* **116**: 281-297

40.������ Lin, S. Y., Johnson, S. M., Abraham, M., Vella, M. C., Pasquinelli, A., Gamberi, C., Gottlieb, E. *et al.* (2003). The C elegans hunchback homolog, hbl-1, controls temporal patterning and is a probable microRNA target. *Dev Cell* **4**: 639-650

41.������ Brennecke, J., Hipfner, D. R., Stark, A., Russell, R. B. & Cohen, S. M. (2003). *bantam* Encodes a Developmentally Regulated microRNA that Controls Cell Proliferation and Regulates the Proapoptotic Gene *hid* in *Drosophila*. *Cell* **113**: 25-36

42.������ Wightman, B., Ha, I. & Ruvkun, G. (1993). Posttranscriptional Regulation of the Heterochronic Gene Lin-14 by Lin-4 Mediates Temporal Pattern-Formation in C-Elegans. *Cell* **75**: 855-862

43.������ Moss, E. G., Lee, R. C. & Ambros, V. (1997). The cold shock domain protein LIN-28 controls developmental timing in C. elegans and is regulated by the lin-4 RNA. *Cell* **88**: 637-646

44.������ Slack, F. J., Basson, M., Liu, Z., Ambros, V., Horvitz, H. R. & Ruvkun, G. (2000). The lin-41 RBCC gene acts in the C. elegans heterochronic pathway between the let-7 regulatory RNA and the LIN-29 transcription factor. *Mol Cell* **5**: 659-669

45.������ Abrahante, J. E., Dau, l. A. L., Li, M., Volk, M. L., Tennessen, J. M., Miller, E. A. & Rougvie, A. E. (2003). The Caenorhabditis elegans hunchback-like gene lin-57/hbl-1 controls developmental time and is regulated by microRNAs. *Dev Cell* **4**: 625-637

46.������ Banerjee, D. & Slack, F. (2002). Control of developmental timing by small temporal RNAs: a paradigm for RNA-mediated regulation of gene expression. *Bioessays* **24**: 119-129

47.������ Rhoades, M. W., Reinhart, B. J., Lim, L. P., Burge, C. B., Bartel, B. & Bartel, D. P. (2002). Prediction of plant microRNA targets. *Cell* **110**: 513-520

48.������� Jones-Rhoades, M. W. & Bartel, D. P. (2004). Computational Identification of Plant MicroRNAs and Their Targets, Including a Stress-Induced miRNA. *Mol Cell* **14**: 787-799

49.������� Robins, H., Li, Y. & Padgett, R. W. (2005). Incorporating structure to predict microRNA targets. *Proc Natl Acad Sci USA* **102**: 4006-4009

50.������� Schwab, R., Palatnik, J. F., Riester, M., Schommer, C., Schmid, M. & Weigel, D. (2005). Specific Effects of MicroRNAs on the Plant Transcriptome. *Dev Cell* **8**: 517-527

51.������� Allen, E., Xie, Z., Gustafson, A. M. & Carrington, J. C. (2005). microRNA-Directed Phasing during Trans-Acting siRNA Biogenesis in Plants. *Cell* **121**: 207-221

52.������� Jackson, A. L., Bartz, S. R., Schelter, J., Kobayshi, S. V., Burchard, J., Mao, M., Li, B. *et al.* (2003). Expression profiling reveals offf-target gene regulation by RNAi. *Nat Biotechnol* **21**: 635-637

53.������� Papp, I., Mette, F., Aufsatz, W., Daxinger, L., Schauer, S. E., Ray, A., van der Winden, J. *et al.* (2003). Evidence for Nuclear Processing of Plant Micro RNA and Short Interfering RNA Precursors. *Plant Physiol* **132**: 1382-1390

54.������� Vargason, J. M., Szittya, G., Burgyan, J. & Tanaka Hall, T. M. (2003). Size selective recognition of siRNA by an RNA silencing suppressor. *Cell* **115**: 799-811

55.������� Ye, K., Malinina, L. & Patel, D. J. (2003). Recognition of small interfering RNA by a viral suppressor of RNA Silencing. *Nature* **426**: 874-878

56.������� Dunoyer, P., Lecellier, C. H., Parizotto, E. A., Himber, C. & Voinnet, O. (2004). Probing the microRNA and small interfering RNA pathways with virus-encoded suppressors of RNA silencing. *Plant Cell* **16**: 1235-1250

57.������� Chapman, E. J., Prokhnevsky, A. I., Gopinath, K., Dolja, V. & Carrington, J. C. (2004). Viral RNA silencing suppressors inhibit the microRNA pathway at an intermediate step. *Genes Dev* **18**: 1179-86

58.������� Mallory, A. C., Reinhart, B. J., Bartel, D., Vance, V. B. & Bowman, L. H. (2002). A viral suppressor of RNA silencing differentially regulates the accumulation of short interfering RNAs and microRNAs in tobacco. *Proc Natl Acad Sci USA* **99**: 15228-15233

59.������� Lecellier, C.-H., Dunoyer, P., Arar, K., Lehmann-Che, J., Eyquem, S., Himber, C., Saib, A. *et al.* (2005). A Cellular MicroRNA Mediates Antiviral Defense in Human Cells. *Science* **308**: 557-560

60.������� Pfeffer, S., Zavolan, M., Grasser, F. A., Chien, M., Russo, J. J., Ju, J., John, B. *et al.* (2004). Identification of Virus-Encoded MicroRNAs. *Science* **304**: 734-736

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Transcription

To make the protein product of a gene, its sequence information needs to be copied from the genomic DNA into messenger RNA. This process, termed transcription, requires an RNA-polymerase. It is initiated at promoter sequences that set the starting point and direction of the transcription. Silencing pathways can either prevent transcription (transcriptional silencing) or interfere with the messenger RNA by degrading it or preventing it from being translated into protein (post-transcriptional silencing).

Expression of a gene

The level of transcription of genomic DNA into RNA is called �RNA expression level� of a gene, while the level of synthesis of the corresponding protein is its �protein expression level�. Post-transcriptional gene silencing occurs in the cytoplasm, following the export of a messenger RNA from the nucleus. Thus, the nuclear RNA expression level is unchanged whereas the cytoplasmic abundance of the targeted RNA is reduced, which in turn leads to a reduced abundance of the protein product. Transcriptional gene silencing, in contrast, reduces or abolishes the transcription of the gene. Thus, the two modes of gene silencing can be distinguished by analysing nuclear and cytoplasmic abundance of the target messenger RNA. �

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Arabidopsis thaliana, A. thaliana

The thale cress *Arabidopsis thaliana* is the preferred model organism for plant genetics and molecular biology. This small weedy plant is easy to grow, has a short life-cycle, produces a large amount of seeds and has a small genome which is now completely sequenced.

RNA interference, RNAi

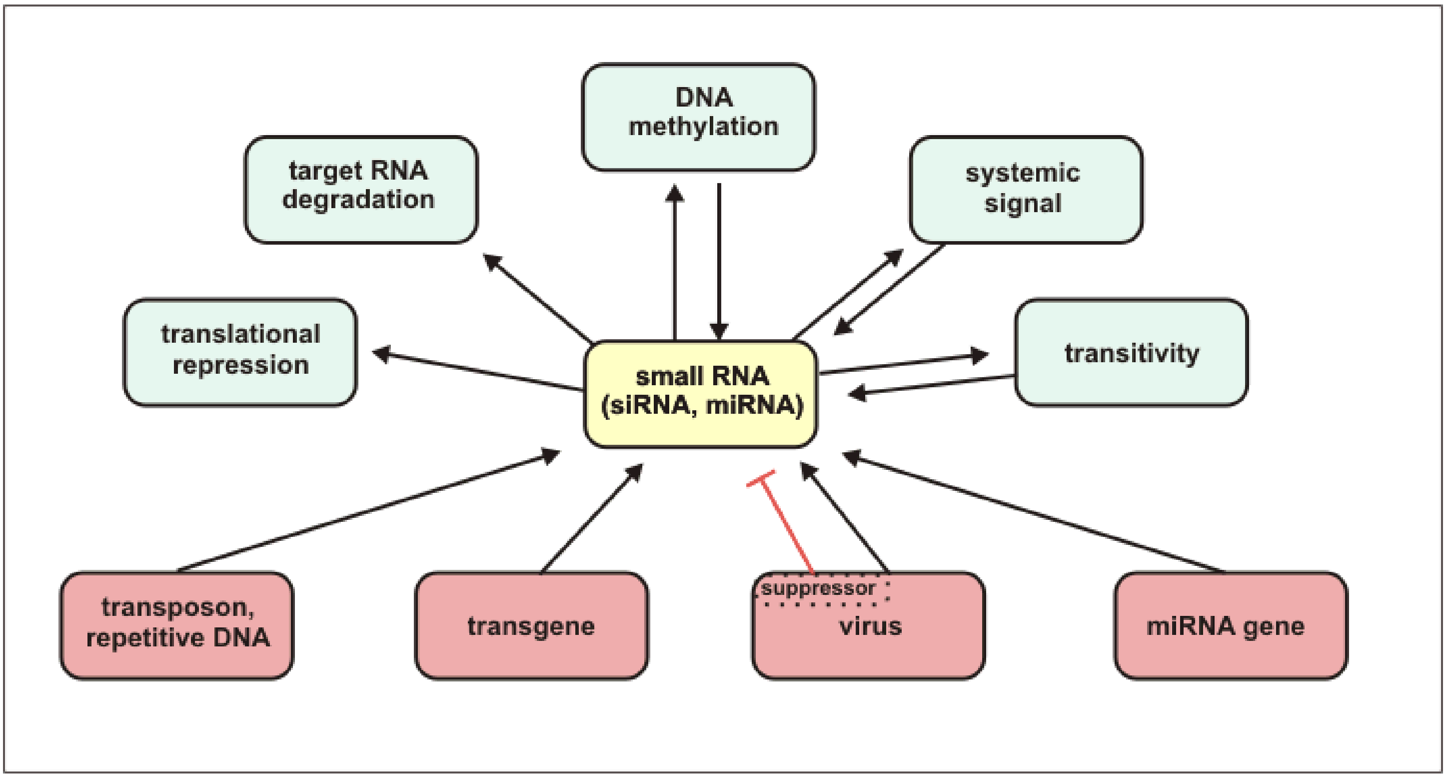
The term �RNA interference� was originally used to describe RNA silencing in animals. It is now often used as a generic term for RNA silencing in all organisms. Constructs that are designed to produce a double-stranded trigger of RNA silencing in transgenic plants are often referred to as RNAi constructs. A co-suppression strategy, in contrast, is based on the expression of copies of the target gene, which give rise to single-stranded RNA.

Post-transcriptional

The term �post-transcriptional� refers to events after the transcription of RNA from its DNA template. Post-transcriptional RNA silencing affects the mature messenger RNA after it has been exported to the cytoplasm (Figure), thus there is no effect of this type of RNA silencing on the transcription rate in the nucleus. Transcriptional silencing, in contrast, inactivates gene expression in the nucleus.

RNA that is transcribed but later degraded in a sequence specific manner is said to be subject to post-transcriptional silencing.

Gene silencing pathways



Overview of gene silencing triggers and effects. Triggers (red) induce various silencing effects (green); however , not all triggers induce all of the effects shown here. Click on image for more detailed information.

Basepairs

DNA and RNA molecules can form double-stranded structures where the nucleobases of one strand are bound to nucleobases of the opposing strand (intermolecular interaction) or of the same strand (intramolecular interaction) by hydrogen bridges. Two nucleobases that are linked to each other by hydrogen bridges are called a �basepair�. Normally, only certain combinations of nucleobases can form basepairs: in DNA, A(denine) pairs with T(hymine) and G(uanine) with C(ytosine). In RNA, U(racil) replaces T(hymine).� However, G often forms a weaker pair with U in RNA, which is referred to as a wobble base pair.

The length of double-stranded DNA molecules is measured in basepairs (bp), whereas the number of unpaired nucleotides (nucleobase plus backbone; nt) is used to measure single-stranded DNA and RNA molecules.

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Genome

The sum of all genes of an organism is its genome.

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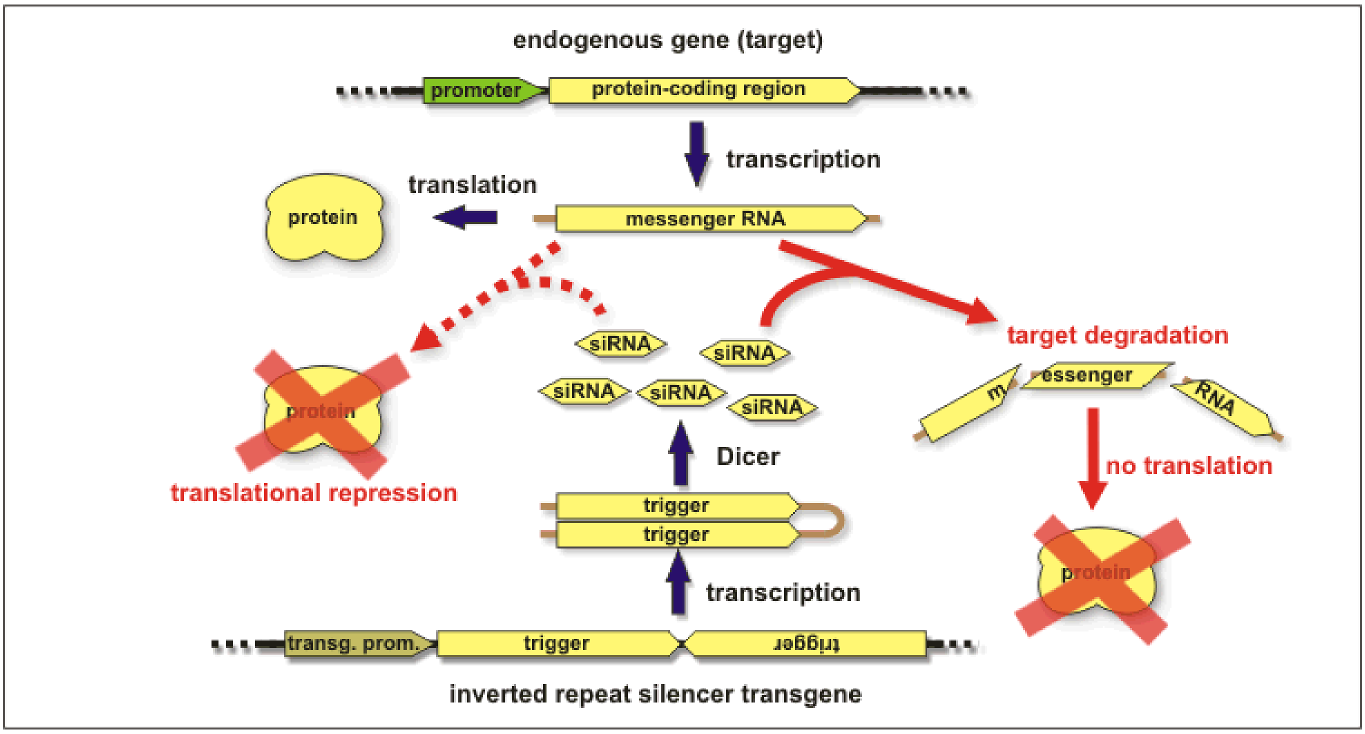
Modes of transgene-induced silencing: post-transcriptional / transcriptional (2 slides)



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Transgene-induced post-transcriptional silencing

In this example, an inverted repeat transgene is expressed under the control of a transgene promoter to generate double-stranded RNA that triggers RNA silencing (see here for detailed Figure).
The sequence of the double-stranded RNA is identical to parts of the protein-coding region of the endogenous target gene. The trigger is converted into siRNAs that induce degradation of the target messenger RNA. No translation into protein can occurr from the degraded RNA, thus target messenger RNA and protein product are absent. If there is insufficient sequence similarity, the siRNA can not induce target degradation but might still interfere with its translation into protein.
In both cases, the transcription rate of the endogenous target gene into messenger RNA is not affected.

Silencing can also be induced by viruses and single stranded RNA (not shown here).

Co-suppression

The term �co-suppression� was coined in the early 1990s to describe the observation that transgenic plants that were transformed with additional copies of a gene sometimes suppress the expression of both, the transgene and the plant gene. We now refer to this phenomenon as post-transcriptional gene silencing or RNA silencing. Co-suppression is still used sometimes to describe a silencing strategy that is based on integration of additional copies of a gene, rather than expression of a double-stranded silencing trigger. The latter is now often referred to as RNAi, a term that was originally used to describe RNA silencing in animals only.

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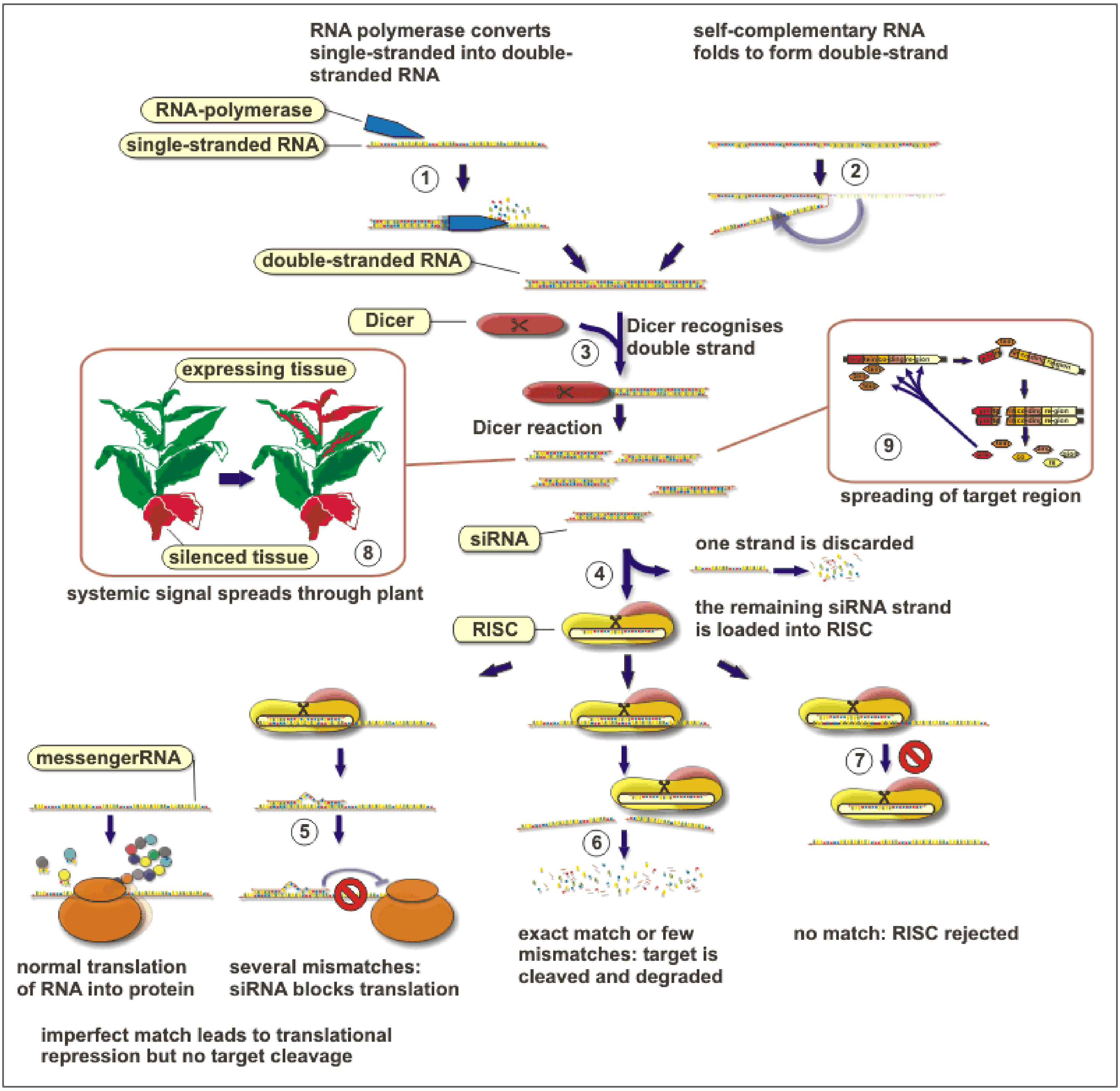
Endogenous gene

An organism�s own genes are called �endogenous genes�. The opposite are foreign, exogenous, genes.

Overview of (post-transcriptional) RNA silencing pathways



This illustration gives an overview of post-trancriptional RNA silencing pathways

RNA silencing is triggered by double-stranded RNA. Single-stranded RNA needs to be converted to the double-sranded form to serve as a trigger. The formation of double-stranded RNA either involves RNA-polymerases or intramolecular interactions. An RNA-polymerase can recognise incorrect RNAs by an unknown mechanism. The "aberrant" RNA is then converted into the double-stranded form by the RNA-polymerase (1). Alternatively, parts of the RNA can be complementary to each other and interact to form a double-stranded "hairpin" structure (2).
The double-stranded RNA formed either way is recognised by Dicer (3). This enzyme processes the long double-stranded RNA into small interfering (si)RNAs, the mediators of sequence specificity in the RNA silencing pathway. These are loaded into the effector complex RISC (RNA-induced silencing complex), where one strand is selectively retained to guide the complex to its target while the other strand is discarded (4).
Depending on the degree of sequence similarity, the siRNA-target interaction can have three different outcomes:

- If the siRNA is imperfectly matched to the target, it may bind but fail to cleave the target. The bound siRNA prevents translation of the target into protein (5).

- If siRNA and target region match perfectly or with very few mismatches, RISC cleaves the target which is subsequently degraded (6).
- If there is insufficient match between siRNA and the probed RNA, RISC is rejected (7).

If the target is a transgene, systemic silencing (8), spreading of the target region within the target gene (transitivity) (9) and methylation of the genomic DNA (not shown here) can be induced.

A more detailed step by step explanation of post-transcriptional silencing pathways can be found here.

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Sense and antisense

Although genomic DNA always consists of two complementary strands, only one of the two is transcribed into messenger RNA that is translated into the protein product of the gene. The strand that is identical in sequence to the messenger RNA is referred to as the sense strand and the complementary strand is called the antisense strand. Sense and antisense RNA, if both expressed, can form double-stranded RNA that triggers the silencing mechanism (see Figure).

The identity of sense and antisense strand is defined by the promoter sequence, which sets the starting point and direction for messenger RNA transcription. Genes can be orientated in both directions on the genomic DNA.

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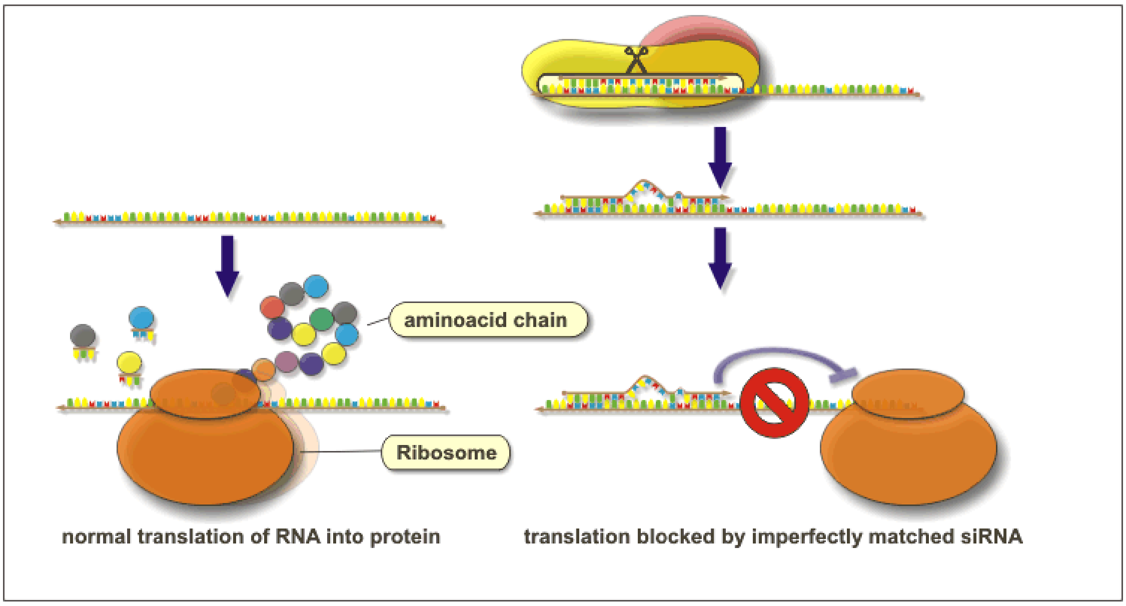
Post-transcriptional RNA silencing triggered by single-stranded RNA (series of 9 slides)



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Step 6 of 9

An imperfectly matched siRNA can induce translational repression instead of target degradation. The siRNA binds to the target but cleavage can not be induced by RISC. Normally, ribosomes (the "protein factories" of the cell) scan the messenger RNA and translate the code of nucleobases into a chain of aminoacids, the building blocks of proteins. By an unknown mechanism, the bound siRNA interferes with this translation process.

Translational repression leads to silencing just as target cleavage does, but it has no effect on the abundance of the target messenger RNA. Therefore, this type of silencing can only be detected if protein abundance is analysed. Although translational repression has been shown in plants, it is far more common in animals.

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Hairpin RNA

Hairpin RNA constructs are used by researchers to trigger RNA silencing in transgenic organisms. They contain a fragment of the target gene (or the entire sequence of the gene) in sense orientation, followed by the same fragment in antisense orientation (Figure). The two fragments are separated by a linker sequence. Because of their physical proximity, the two complementary fragments of the resulting messenger RNA can easily interact to form a double-stranded substrate for Dicer, thus triggering RNA silencing against the target gene [1,2] . When they were first introduced, hairpin constructs were difficult to make but many tools and techniques are now available to facilitate their construction [3,4] .

Literature

1.�������� Wesley, S. V., Helliwell, C. A., Smith, N. A., Wang, M., Rouse, D. T., Liu, Q., Gooding, P. S. *et al.* (2001). Construct design for efficient, effective and high-throughput gene silencing in plants. *Plant J* **27**: 581-590

2.�������� Smith, N. A., Singh, S. P., Wang, M. B., Stoutjesdijk, P. A., Green, A. G. & Waterhouse, P. M. (2000). Total silencing by intron-spliced hairpin RNAs. *Nature* **407**: 319-320

3.�������� Pawloski, L. C., Deal, R. B., McKinney, E. C., Burgos-Rivera, B. & Meagher, R. B. (2005). Inverted Repeat PCR for the Rapid Assembly of Constructs to Induce RNA Interference. *Plant Cell Physiol* **Advance Access published online on 24/08/05**

4.�������� Wesley, S. V., Helliwell, C., Wang, M. B. & Waterhouse, P. (2004). Posttranscriptional gene silencing in plants. *Methods Mol Biol* **265**: 117-129

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Short-hairpin (sh)RNA

Transforming plants with constructs that direct the expression of long double-stranded RNA (so called �hairpin� constructs) efficiently triggers RNA silencing (see Figure). However, this approach cannot be used in mammalian cells where long double-stranded RNAs trigger a cytotoxic reaction that leads to cell death [1] . This reaction, mediated by the interferon system, protects the organism from RNA viruses by sacrificing the infected cell, thus preventing spreading of the virus [2] . Double-stranded RNAs shorter than 30 nucleotides do not trigger the interferon response, therefore scientists have developed artificially produced siRNAs and protocols for their delivery into mammalian cells to efficiently induce RNA silencing [3] . However, siRNA-induced silencing is short-lived and cannot be used to study long-term effects. For this reason, constructs were developed to directly express siRNA-like molecules in cells [4] . These constructs use RNA-polymerase 3 to express a short hairpin (sh)RNA. This polymerase is specialised to transcribe short templates with a precisely defined termination signal. The resulting transcript is about twice as long as the mature siRNA and folds back upon itself to form a double-stranded precursor with one end exhibiting the 2-nucleotide overhang that is typical for siRNAs, while the other end forms a bulge. Dicer recognises the open end of this structure and excises the mature siRNA, thus producing a single siRNA from each transcript [5,6] .

Recently, shRNA constructs have been demonstrated to function in plants as well [7] . The small size of shRNAs makes them a preferred tool compared to long double-stranded RNAs, since the latter are processed into a pool of siRNAs, many of which might bind to unforeseen targets. In contrast, shRNAs can be tailored to specifically match the target gene, thus minimising off-target effects. Off-target effects might be further reduced by using shorter versions of shRNAs as shown in a recent study in mammalian cells [6] .

Literature

1.�������� Hunter, T., Hun, T., Jackson, R. J. & Robertson, H. D. (1975). The characteristics of inhibition of protein synthesis by double-stranded ribonucleic acid in reticulocyte lysates. *J Biol Chem* **250**: 409-417

2.�������� Stark, G. R., Kerr, I. M., Williams, B. R. G., Silverman, R. H. & Schreiber, R. D. (1998). How cells respond to interferons. *Ann Rev Biochem* **67**: 227-264

3.�������� Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. & Tuschl, T. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**: 494-498

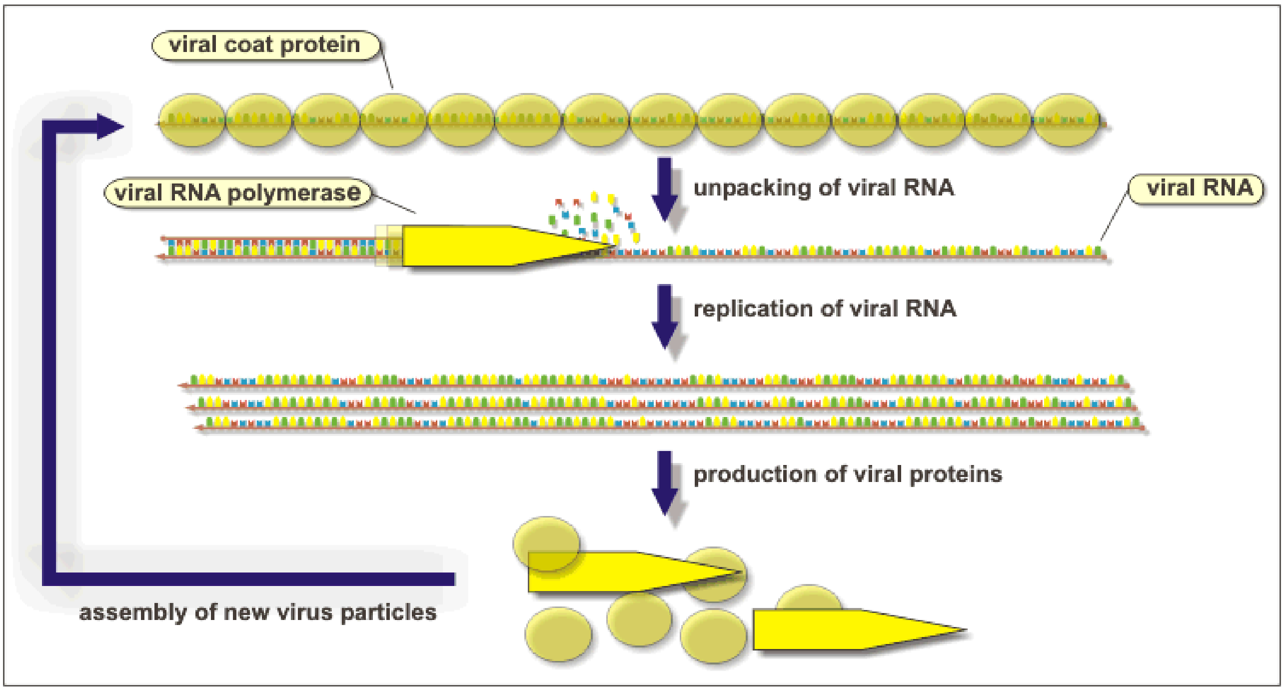
4.�������� Brummelkamp, T. R., Bernards, R. & Agami, R. (2002). A system for stable expression of short interfering RNAs in mammalian cells. *Science* **296**: 550-553

5.�������� Kim, D. H., Behlke, M. A., Rose, S. D., Chang, M. S., Choi, S. & Rossi, J. J. (2005). Synthetic dsRNA Dicer substrates enhance RNAi potency and efficacy. *Nat Biotechnol* **23**: 222-226

6.�������� Siolas, D., Lerner, C., Burchard, J., Ge, W., Linsley, P. S., Paddison, P. J., Hannon, G. J. *et al.* (2005). Synthetic shRNAs as potent RNAi triggers. *Nat Biotechnol* **23**: 227-231

7.�������� Lu, S., Shi, R., Tsao, C. C., Yi, X., Li, L. & Chiang, V. L. (2004). RNA silencing in plants by the expression of siRNA duplexes. *Nucleic Acids Res* **32**: e171

Viruses as triggers and targets of RNA silencing (series of 7 slides)



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Step 1 of 7

This series of images shows how viruses trigger RNA silencing.
The genome of most plant virus consists of one or more molecules of single-stranded RNA. DNA (single or double stranded) and double-stranded RNA genomes are found less frequently among plant viruses but are common among viruses infecting other organisms.
After entering the plant cell, the genomic RNA is released from its protein coat. A virus-encoded RNA-polymerse replicates the RNA genome, which is translated to produce viral proteins. To close the viral "life"-cycle, the new copies of the genome are re-packed with coat protein units to yield infectious viral particles.

Dicer (DCR) and Dicer-like (DCL)

Dicer (DCR) is the name given to a class of key enzymes in RNA silencing in animals and fungi, which process long double-stranded RNA into short siRNAs. The homologous enzymes in plants were later named Dicer-like (DCL). These enzymes contain RNA binding, RNA unwinding and RNA cleaving domains [1] . In the fruitfly *Drosophila melanogaster*, Dicer enzymes have also been shown to assist in assembling the silencing effector complex RISC and to �hand over� the siRNAs they produce to this complex, which is why they are required even if "ready-made" siRNAs are delivered to a cell [2] .

There are four DCL proteins in the model plant *Arabidopsis thaliana* (DCL1, 2, 3 and 4). Mammals encode only a single Dicer in their genomes while two are found in the fruitfly *Drosophila melanogaster*.

Literature

1.�������� Schauer, S. E., Jacobsen, S. E., Meinke, D. W. & Ray, A. (2002). DICER-LIKE1: blind men and elephants in *Arabidopsis* development. *Trends Plant Sci* **7**: 487-491

2.�������� Sontheimer, E. J. (2005). Assembly and Function of RNA Silencing Complexes. *Nat Rev Mol Cell Biol* **6**: 127-138

RNA-induced silencing complex (RISC)

RISC is a key player of post-transcriptional RNA silencing. It was first identified� in the fruitfly *Drosophila melanogaster* as an RNA-directed nuclease that binds siRNAs as a guide to identify target sequences [1] . RISC is a complex of proteins with varying protein-composition in different organisms [2,3,4,5] . In *Drosophila melanogaster*, several components of RISC have recently been identified, including an ARGONAUTE protein and an RNA helicase (a protein that unwinds RNA) [6] . ARGONAUTE proteins are an essential part of RISC in different organisms [2,4,7] . The plant RISC complex has not yet been isolated but an ARGONAUTE protein has been shown recently to perform the siRNA-guided target cleavage that is thought to be the core function of RISC [8,9] . The RISC activity could be carried out entirely by this ARGOANUTE protein, so it is possible that there might not be a RISC complex as such in plants [8] .

An overview of pathways involving RISC can be found here.

Literature

1.�������� Hammond, S. M., Bernstein, E., Beach, D. & Hannon, G. (2000). An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cell extracts. *Nature* **404**: 293-296

2.�������� Hammond, S. M., Boettcher, S., Caudy, A. A., Kobayashi, R. & Hannon, G. J. (2001). Argonaute2, a link between genetic and biochemical analyses of RNAi. *Science* **293**: 1146-1150

3.�������� Nykanen, A., Haley, B. & Zamore, P. D. (2001). ATP requirements and small interfering RNA structure in the RNA interference pathway. *Cell* **107**: 309-321

4.�������� Martinez, J., Patkaniowska, A., Urlaub, H., Luhrmann, R. & Tuschl, T. (2002). Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. *Cell* **110**: 563-574

5.�������� Pham, J. W., Pellino, J. L., Lee, Y. S., Carthew, R. W. & Sontheimer, E. J. (2004). A Dicer-2-Dependent 80S Complex Cleaves Targeted mRNAs during RNAi in *Drosphila*. *Cell* **117**: 83-94

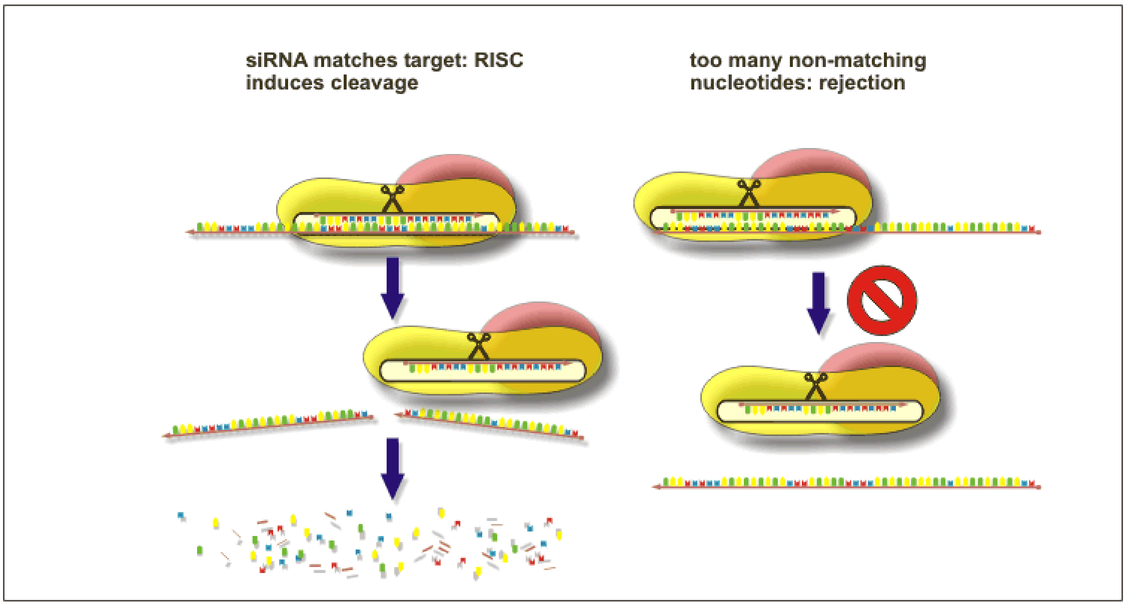
6.�������� Siomi, M. C. & Siom, i. H. (2005). Identification of Components of RNAi Pathways Using the Tandem Affinity Purification Method. *Methods Mol Biol* **309**: 1-10

7.�������� Hutvagner, G. & Zamore, P. D. (2002). A microRNA in a multiple-turnover RNAi enzyme complex. *Science* **297**: 2056-2060

8.�������� Baumberger, N. & Baulcombe, D. C. (2005). Arabidopsis ARGONAUTE1 is an RNA Slicer that selectively recruits microRNAs and short interfering RNAs. *Proc Natl Acad Sci USA* **102**: 11928-11933

9.�������� Qi, Y., Denli, A. M. & Hannon, G. J. (2005). Biochemical specialization within Arabidopsis RNA silencing pathways. *Mol Cell* **19**: 421-428

Post-transcriptional RNA silencing triggered by single-stranded RNA (series of 9 slides)



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Step 5 of 9

RISC identifies target RNAs by using the siRNA as a probe. If siRNA and target match sufficiently, RISC cleaves the target RNA, which is subsequently degraded. RISC is then free to seek out more targets using the same siRNA probe. If siRNA and target do not match sufficiently, RISC is rejected and no cleavage occurs. In some cases, an imperfectly matched siRNA can still prevent translation of the target into a protein as shown on the next slide.

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ARGONAUTE (AGO) proteins

All organisms capable of carrying out RNA silencing possess at least one member of the extensive and highly conserved family of ARGONAUTE proteins. The first one to be identified was the *Arabidopsis* ARGONAUTE1 (AGO1) and the severe developmental defects in plants lacking a functional version of this protein [1] are now known to be caused by a disruption in microRNA-mediated gene-regulation for which AGO1 is required [2] . ARGONAUTE proteins are essential components of the silencing effector complexes (RISC and RITS), although the exact functions of these proteins are just beginning to emerge [3,4,5,6,7,8,9,10,11] .

�Recently, members of the ARGONAUTE protein family have been characterized in bacteria where they carry out guided RNA degradation [12] although in eubacteria, unlike in higher organisms, the guide seems to be a single-stranded DNA instead of a small RNA [12] .

The genomes of many organisms encode several members of the ARGONAUTE family: there are 27 ARGONAUTEs in the nematode *C. elegans*, 10 in the plant *A. thaliana*, 8 in humans, 5 in the fruitfly *D. melanogaster* and 2 in the fungus *N. crassa* [13] . The large number of ARGONAUTE proteins in some organisms might indicate that there are more different silencing pathways than we know of today.

Literature

1.�������� Bohmert, K., Camus, I., Bellini, C., Bouchez, D., Caboche, M. & Benning, C. (1998). *AGO1* defines a novel locus of *Arabidopsis* controlling leaf development. *EMBO J* **17**: 170-180

2.�������� Vaucheret, H., Vazquez, F., Crete, P. & Bartel, D. P. (2004). The action of ARGONAUTE1 in the miRNA pathway and its regulation by the miRNA pathway are crucial for plant development. *Genes Dev* **18**: 1187-1197

3.�������� Tabara, H., Sarkissian, M., Kelly, W. G., Fleenor, J., Grishok, A., Timmons, L., Fire, A. *et al.* (1999). The rde-1 gene, RNA interference and transposon silencing in *C. elegans*. *Cell* **99**: 123-132

4.�������� Tabara, H., Yigit, E., Siomi, H. & Mello, C. C. (2002). The dsRNA binding protein RDE-4 interacts with RDE-1, DCR-1, and a DExX-box helicase to direct RNAi in C-elegans. *Cell* **109**: 861-871

5.�������� Hammond, S. M., Boettcher, S., Caudy, A. A., Kobayashi, R. & Hannon, G. J. (2001). Argonaute2, a link between genetic and biochemical analyses of RNAi. *Science* **293**: 1146-1150

6.�������� Hutvagner, G., McLachlan, J., Pasquinelli, A. E., Balint, E., Tuschl, T. & Zamore, P. D. (2001). A cellular function for the RNA-interference enzyme Dicer in the maturation of the *let-7* small temporal RNA. *Science* **293**: 834-838

7.�������� Catalanotto, C., Azzalin, G., Macino, G. & Cogoni, C. (2002). Involvement of small RNAs and role of the *qde* genes in the gene silencing pathway in *Neurospora*. *Genes Dev* **16**: 790-795

8.�������� Martinez, J., Patkaniowska, A., Urlaub, H., Luhrmann, R. & Tuschl, T. (2002). Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. *Cell* **110**: 563-574

9.�������� Mourelatos, Z., Dostie, J., Paushkin, S., Sharma, A., Charroux, B., Abel, L., Rappsilber, J. *et al.* (2002). miRNPs: a novel class of ribonucleoproteins containing numerous microRNAs. *Genes Dev* **16**: 720-728

10.������ Djikeng, A., Shi, H., Tschudi, C., Shen, S. & Ullu, E. (2003). An siRNA ribonucleoprotein is found associated with polyribosomes in Trypanosoma brucei. *RNA* **9**: 802-808

11.������ Shi, H., Djikeng, A., Tschudi, C. & Ullu, E. (2004). Argonaute protein in the early divergent eukaryote Trypanosoma brucei: control of small interfering RNA accumulation and retroposon transcript abundance. *Mol Cell* **24**: 420-427

12.������ Yuan, Y. R., Pei, Y., Ma, J. B., Kuryavyi, V., Zhadina, M., Meister, G., Chen, H. Y. *et al.* (2005). Crystal Structure of A. aeolicus Argonaute, a Site-Specific DNA-Guided Endoribonuclease, Provides Insights into RISC-Mediated mRNA Cleavage. *Mol Cell* **19**: 405-419

13.������ Carmell, M. A., Xuan, Z., Zhang, M. & Hannon, G. J. (2002). The Argonaute family: tentacles that reach into RNAi, developmental control, stem cell maintenance, and tumorigenesis. *Genes Dev* **16**: 2733-2742

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HEN1

The *Arabidopsis* HEN1 (HUA ENHANCER1) protein is involved in miRNA biosynthesis [1,2] and in RNA silencing pathways that involve an RNA-dependent RNA-polymerase activity [2] . Neither HEN1 nor HYL1 are absolutely required for miRNA biosynthesis but if both are missing the plant is infertile, suggesting a synergistic effect of these two proteins [3] . HEN1 is an RNA methyltransferase that modifies the 3� end of miRNAs and siRNAs [4,5] . Unmethylated ends of miRNAs and siRNAs are marked out for degradation [5,6] . Methylation might also prevent miRNAs from being used as primers, �thus disabling undesirable miRNA-induced transitivity on endogenous targets [4] .

Literature

1.�������� Park, W., Li, J., Song, R., Messing, J. & Chen, X. (2002). CARPEL FACTORY. a Dicer homolog, and HEN1, a novel protein, act in microRNA metabolism in *Arabidopsis thaliana*. *Curr Biol* **12**: 1484-1495

2.�������� Boutet, S., Vazquez, F., Liu, J., Beclin, C., Morel, J.-B., Crete, P., Chen, X. *et al.* (2003). *Arabidopsis HEN1*: A Genetic Link between Endogenous miRNA Controlling Development and siRNA Controlling Transgene Silencing and Virus Resistance. *Curr Biol* **13**: 843-848

3.�������� Vazquez, F., Gasciolli, V., Crete, P. & Vaucheret, H. (2004). The Nuclear dsRNA Binding Protein HYL1 is Required for MicroRNA Accumulation and Plant Development, but not Posttranscriptional Transgene Silencing. *Curr Biol* **14**: 346-351

4.�������� Yu, B., Yang, Z., Li, J., Minakhina, S., Yang, M., Padgett, R. W., Steward, R. *et al.* (2005). Methylation as a Crucial Step in Plant microRNA Biogenesis. *Science* **307**: 932-935

5.�������� Li, J., Yang, Z., Yu, B., Liu, J. & Chen, X. (2005). Methylation Protects miRNAs and siRNAs from a 3'-End Uridylation Activity in Arabidopsis. *Curr Biol* **15**: 1501-1507

6.�������� Shen, B. & Goodman, H. M. (2004). Uridine Addition After MicroRNA-Directed Cleavage. *Science* **306**: 997

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Inverted repeat

The term �inverted repeat� is frequently used in the scientific literature on RNA silencing. In this context it refers to a DNA or RNA sequence that is self-complementary. This is achieved by a sequence that is followed, either directly adjoining or interrupted by a spacer sequence, by its complement in reverse orientation (see Figure). Inverted repeats are often used to construct silencer transgenes, because the resulting double-stranded RNA is a very potent trigger of RNA silencing.

Promoter

Promoter sequences precede every protein-coding gene. They are not part of the final messenger RNA but they control its spatial and temporal expression pattern by serving as a recognition sequence for DNA-binding components of the transcription machinery. Genes are transcribed in a directional manner, thus the promoter sets the starting point and the direction in which transcription is to proceed.

Caenorhabditis elegans, nematodes

Nematodes, or roundworms, are structurally simple organisms. They are probably the most numerous multicellular animals on earth. The nematode *Caenorhabditis elegans* is a very important model organism for molecular biologists. It is very easy to trigger RNA silencing in *C. elegans* because the worm feeds on bacteria which can be engineered to produce double-stranded RNA. These molecules are ingested through the worms� gut cells and trigger RNA silencing throughout the animal [1] .

Literature

1.��������� Kamath, R. S., Martinez-Campos, M., Zipperlen, P., Fraser, A. G. & Ahringer, J. (2000). Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in Caenorhabditis elegans. *Genome Biol* **2**: 231-237

RNA-dependent RNA-polymerase

An RNA-dependent RNA-polymerase (RdRp) is an enzyme that uses RNA as a template to produce a second (complementary) strand of RNA.
RNA viruses produce their own RNA-dependent RNA-polymerases to multiply their genomic RNA. Although they have been known for a long time [1,2,3,4], the function of plant-expressed RNA-dependent RNA-polymerases remained unclear until the discovery of RNA silencing. The *Arabidopsis thaliana* genome encodes six RNA-dependent RNA-polymerases, termed RDR1, RDR2, RDR3, RDR4, RDR5 and RDR6. Some of the functions of RDR1, 2 and 6 in RNA silencing are known but it is still unclear whether or not RDR3, 4 and 5 are actually functional and what their roles in RNA silencing could be.

Literature

1.�������� Astier-Manifacier, S. & Cornuet, P. (1971). RNA-dependent RNA polymerase in Chinese cabbage. *Biochim Biophys Acta* **232**: 484-493

2.�������� Schiebel, W., Haas, B., Marinkovic, S., Klanner, A. & Sanger, H. L. (1993). RNA-directed RNA polymerase from tomato leaves.� I. Purification and physical properties. *Journal of Biological Chemistry* **268**: 11851-11857

3.�������� Schiebel, W., Haas, B., Marinkovic, S., Klanner, A. & Sanger, H. L. (1993). RNA-directed RNA polymerase from tomato leaves.� II. Catalytic �*in vitro* properties. *Journal of Biological Chemistry* **268**: 11858-11867

4.�������� Schiebel, W., Pelissier, T., Reidel, L., Thalmeir, S., Schiebel, R., Kempe, D., Lottspeich, F. *et al.* (1998). Isolation of an RNA-directed RNA polymerase-specific cDNA clone from tomato. *Plant Cell* **10**: 2087-2102

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Complement, complemantary DNA/RNA strand

Two strands of DNA or RNA that bind to each other to form a double-stranded molecule are not copies of each other � they are complementary to each other. DNA and RNA encode their information in a sequence of the four nucleobases (A)denine, (T)hymine, (G)uanine and (C)ytosine. In RNA (U)racil replaces (T)hymine. These nucleobases can establish basepairs, where a nucleobase of one strand is bound to a nucleobase of the opposing strand. In such basepairs, A is normally paired with T (or U in RNA) and G with C. Therefore, knowing the sequence of one strand is sufficient to derive the sequence of the opposing strand provided they are bound to each other over their entire length. The level of complementarity between two DNA or RNA strands is a measure for the amount of possible basepairs that can be established between the two.

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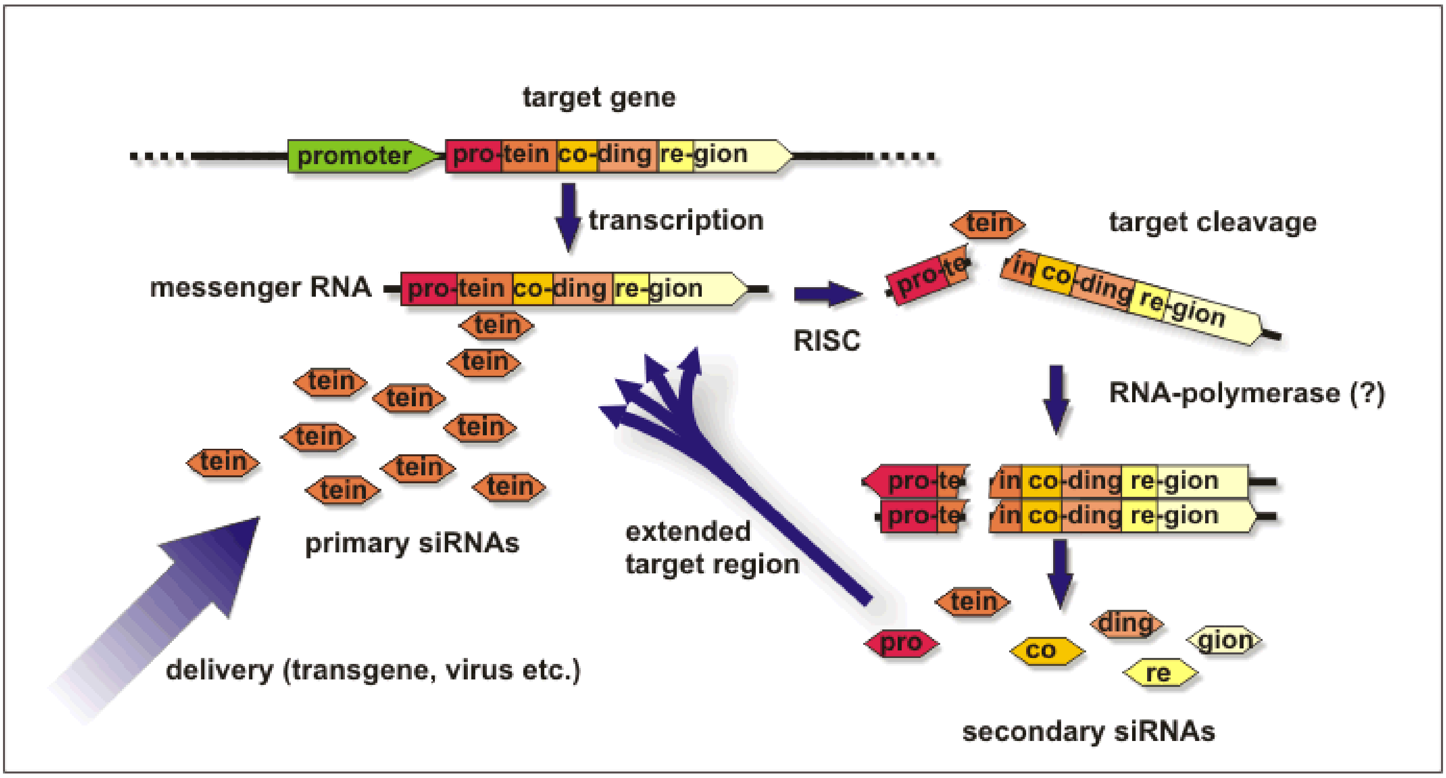
Transitivity (spreading of target region)



Transitivity

The target region of RNA silencing can be extended in a process known as "transitivity". In this example, all siRNAs delivered initially (primary siRNAs) correspond to the same part of the protein coding region of the target gene (labelled "tein"). Silencing is induced and the target messenger RNA is cleaved within the "tein" region by the effector complex RISC, programmed with the primary siRNAs.

It is not known how exactly the target region is extended. One possibility (shown here) is that the two fragments that result from the cleavage of the target messenger RNA are recognised as an "aberrant" RNA by an RNA-polymerase, which converts the fragments into doube-stranded RNA (see details). This new double-stranded RNA encompasses the entire messenger RNA and it is processed by Dicer into siRNAs. These secondary siRNAs target all regions of the messenger RNA.

Transitivity is also linked to methylation, which may indicate a different mechanism involving transcription of double-stranded RNA from the genomic copy of the gene. Alternatively, primary siRNAs might guide the RNA-polymerase directly to its target and prime the polymerase reaction (not shown here).

So far, transitivity has only been observed when transgenes are targets of RNA silencing. Endogenous genes seem to be protected from this process.

SiRNAs can either be delivered directly or they can be produced from transgenes or viruses.

Transitivity (Figure)

In nematodes and plants, triggering RNA-silencing against one region of a transgenic messenger RNA results in the formation of siRNAs corresponding not only to that region but to the entire messenger RNA. This spreading of targeting beyond the initial trigger region is known as transitivity and requires the activity of an RNA-dependent RNA-polymerase [1,2,3,4,5] . The exact mechanism is not clear yet but it is conceivable that the RNA-polymerase recognises siRNAs that are bound to their target RNAs as primers. The result would be a double-stranded RNA that extends beyond the initially targeted region to which the siRNA was bound. This extended double-stranded RNA can be processed into siRNAs. Because of the unidirectional mode of action of all polymerases, transitivity should extend the target region exclusively towards the 5� end of the template. This is indeed observed in nematodes [2,4,6] . However, plants surprisingly exhibit transitivity towards both ends of the template [1,3,5,7] . To explain this phenomenon it has been suggested that the plant RNA-polymerase responsible for transitivity mainly acts in an unprimed mode, i.e. it recognises the fragments that arise from the initial siRNA-directed target cleavage and converts them into double-stranded RNA beginning from the 3� ends [3,7] . Alternatively, there could be small amounts of antisense transcript corresponding to the target RNA, which would allow a primer-dependent polymerase reaction that would extend the target towards the 3� end of the sense transcript [3] . Recent data do indeed suggest that a large part of plant genomes may be transcribed in the antisense orientation [8] . Biochemical studies have revealed both primer-dependent and primer-independent RNA-polymerase activities in plants and fungi [9,10] .
Transitivity in plants affects transgenes only, while endogenous targets seem to be protected from this process by an unknown mechanism [3,11] .

Literature

1.��������� Klahre, U., Crete, P., Leuenberger, S. A., Iglesias, V. A. & Meins, F. (2002). High molecular weight RNAs and small interfering RNAs induce systemic posttranscriptional gene silencing in plants. *Proc Natl Acad Sci USA* **99**: 11981-11986

2.��������� Sijen, T., Fleenor, J., Simmer, F., Thijssen, K. L., Parrish, S., Timmons, L., Plasterk, R. H. A. *et al.* (2001). On the role of RNA amplification in dsRNA-triggered gene silencing. *Cell* **107**: 465-476

3.��������� Vaistij, F. E., Jones, L. & Baulcombe, D. C. (2002). Spreading of RNA targeting and DNA methylation in RNA silencing requires transcription of the target gene and a putative RNA-dependent RNA polymerase. *Plant Cell* **14**: 857-867

4.��������� Alder, M. N., Dames, S., Gaudet, J. & Mango, S. E. (2003). Gene silencing in Caenorhabditis elegans by transitive RNA interference. *RNA* **9**: 25-32

5.��������� Voinnet, O., Vain, P., Angell, S. & Baulcombe, D. C. (1998). Systemic spread of sequence-specific transgene RNA degradation is initiated by localised introduction of ectopic promoterless DNA. *Cell* **95**: 177-187

6.��������� Lipardi, C., Wei, Q. & Paterson, B. M. (2001). RNAi as random degradative PCR: siRNA primers convert mRNA into dsRNAs that are degraded to generate new siRNAs. *Cell* **107**: 297-307

7.��������� Petersen, B. O. & Albrechtsen, M. (2005). Evidence implying only unprimed RdRP activity during transitive gene silencing in plants. *Plant Mol Biol* **58**: 575-583

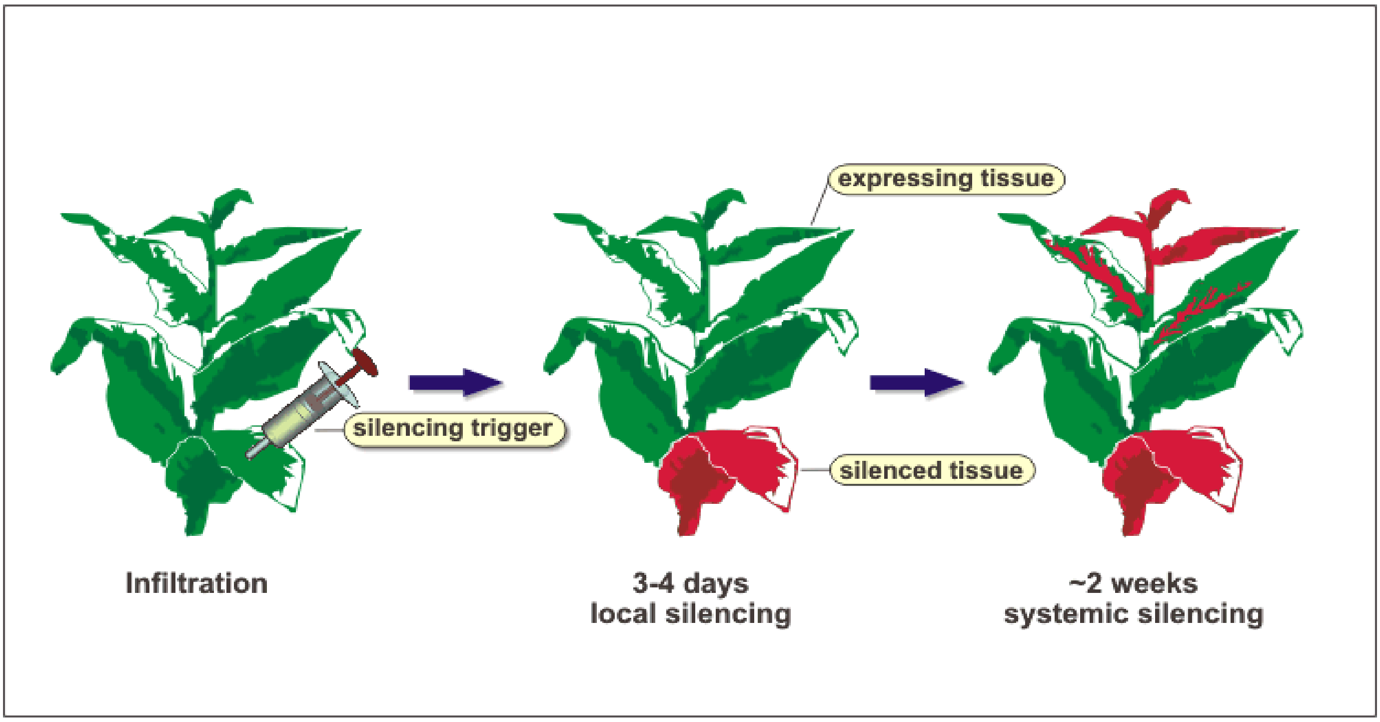
8.��������� Yamada, K., Lim, J., Dale, J. M., Chen, H., Shinn, P., Palm, C. J., Southwick, A. M. *et al.* (2003). Empirical analysis of transcriptional activity in the Arabidopsis genome. *Science* **302**: 842-846

9.��������� Tang, G., Reinhart, B. J., Bartel, D. P. & Zamore, P. D. (2003). A biochemical framework for RNA silencing in plants. *Genes Dev* **17**: 49-63

10.������ Makeyev, E. V. & Bamford, D. H. (2002). Cellular RNA-dependent RNA polymerase involved in posttranscriptional gene silencing has two distinct activity modes. *Mol Cell* **10**: 1417-1427

11.������ Han, Y. H., Grierson, D. & Han, Y. H. (2002). Relationship between small antisense RNAs and aberrant RNAs associated with sense transgene mediated gene silencing in tomato. *Plant J* **29**: 509-519

Systemic silencing signals (2 slides)



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Induction of systemic silencing by "agro-infiltration"

Plant RNA silencing involves a mobile signal that spreads through the plant to cause systemic silencing. In a standard assay a reporter transgene, e.g. the jellyfish green fluorescent protein, is silenced locally by infiltrating a leaf with a bacterium culture that expresses a silencing trigger. The bacterium used is *Agrobacterium tumefaciens*, which is why this widely used procedure is known as "agro-infiltration".
Within a few days, the reporter gene is silenced (red area) in the infiltrated leaf. A signal of unknown identity spreads through the vasculature of the plant and triggers systemic silencing of the same target gene first in newly emerging leaves and later in the enitre plant. Since the signal retains the sequence specificity of the initial silencing it is often thought to be siRNA, probably associated with a transport protein.

The systemic spreading of a silencing signal resembles the long-range movement of plant viruses. Since they are targets of the silencing machienery, viruses must either outrace the silencing signal or inactivate it. Consequently, some viral silencing suppressor proteins interfere specifically with the signal step of RNA silencing.

Only transgenes and pathogens can be targeted by a systemic silencing signal but it is not known yet how the plant's own genes are protected from becoming systemically silenced.

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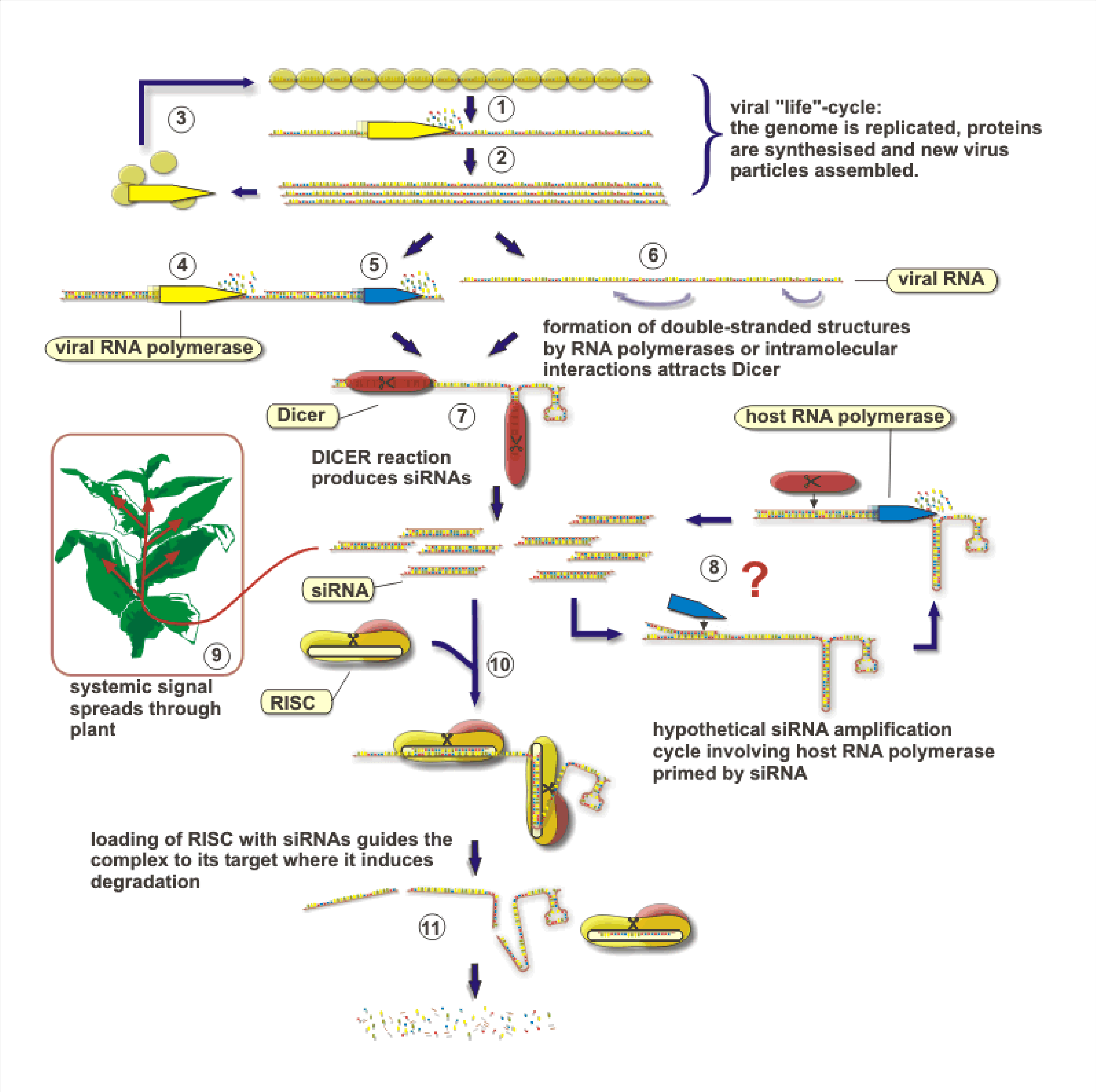
Viruses as triggers and targets of RNA silencing - overview



This illustration gives an overview of the RNA silencing pathway triggered by a single-stranded RNA virus.

The incoming virus genome is unpacked (1), replicated (2) and re-packed (3) to complete its "life"-cycle. This requires the activity of a viral RNA-polymerase which results in the temporary formation of double-stranded RNA (4). In addition, host (plant) encoded RNA-polymerases are thought to contribute to the formation of double-stranded viral RNA (5). Single-stranded viral RNA can also form partially double-stranded structures due to intramolecular interactions between regions with complementary sequences (6).

Double-stranded RNA is recognised by Dicer which processes the viral RNA into siRNAs (7). These might feed into a hypothetical siRNA amplification cycle, involving a host RNA-polymerase (8).

Triggering silencing locally induces a systemic silencing signal that spreads through the plant to "immunise" the entire plant against the virus (9). This signal is thought to involve siRNA.

SiRNAs are loaded into the effector complex RISC to identify the targets of RNA silencing (10). Viral RNA (and any other RNA with sufficient sequence similarity) is cleaved by components of RISC and subsequently degraded (11).

A more detailed step by step explanation can be found here.

Suppressors of gene silencing

One of the major roles of RNA silencing in plants is to provide a defence system against viruses. Therefore, viruses are under strong selection pressure to develop ways of evading or counter-acting the silencing machinery. Many, if not all, plant viruses and at least some animal viruses consequently encode proteins that suppress gene silencing [1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18] . Viral suppressor proteins have apparently evolved independently of each other because they exhibit a broad spectrum of activities and interactions with the host silencing machinery. The ability of viral silencing suppressors to interfere with different steps of gene silencing pathways make them ideal tools to dissect these pathways [1,5,17,19,20,21,22,23,24,25,26,27,28,29,30,31,32,33,34,35,36,37,38] .

Among the best characterised suppressors are the potyvirus HC/Pro and the tombusvirus P19 protein. Resolving the crystal structure of the latter made it possible to see a viral silencing suppressor in action, showing that it specifically binds to siRNAs which fit into a �mould� formed by two interacting copies of the protein [5,25] . The P19 protein is thought to validate the identity of the bound RNA by probing for the 2 nucleotide overhangs that are typical for siRNAs. The suppressor functions by depleting the cell of the siRNAs that would otherwise target the virus for degradation.

In addition to viral-encoded suppressors of gene silencing, plants seem to have their own suppressors but their role in the diverse silencing pathways is not clear yet [39] .

Literature

1.�������� Moissiard, G. & Voinnet, O. (2004). Viral suppression of RNA silencing in plants. *Mol Plant Pathol* **5**: 71-82

2.�������� Reed, J. C., Kasschau, K. D., Prokhnevsky, A. I., Gopinath, K., Pogue, G. P., Carrington, J. C. & Dolja, V. V. (2003). Suppressor of RNA silencing encoded by Beet yellows virus. *Virology* **306**: 203-209

3.�������� Vanitharani, R., Chellappan, P., Pita, J. S. & Fauquet, C. M. (2004). Differential Roles of AC2 and AC4 of Cassava Geminiviruses in Mediating Synergism and Suppression of Posttranscriptional Gene Silencing. *J Virol* **78**: 9487-9498

4.�������� Merai, Z., Kerenyi, Z., Molnar, A., Barta, A., Valoczi, A., Bisztray, G., Havelda, Z. *et al.* (2005). Aureusvirus P14 Is an Efficient RNA Silencing Suppressor That Binds Double-Stranded RNAs without Size Specificity. *J Virol* **79**: 7217-7226

5.�������� Vargason, J. M., Szittya, G., Burgyan, J. & Tanaka Hall, T. M. (2003). Size selective recognition of siRNA by an RNA silencing suppressor. *Cell* **115**: 799-811

6.�������� Brigneti, G., Voinnet, O., Li, W. X., Ji, L. H., Ding, S. W. & Baulcombe, D. C. (1998). Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. *EMBO J* **17**: 6739-6746

7.�������� Silhavy, D., Molnar, A., Lucioli, A., Szittya, G., Hornyik, C., Tavazza, M. & Burgyan, J. (2002). A viral protein suppresses RNA silencing and binds silencing-generated, 21-to 25-nucleotide doube-stranded RNAs. *EMBO J* **21**: 3070-3080

8.�������� Kubota, K., Tsuda, S., Tamai, A. & Meshi, T. (2003). Tomato mosaic virus replication protein suppresses virus- targeted posttranscriptional gene silencing. *J Virol* **77**: 11016-11026

9.�������� Ding, X. S., Liu, J. Z., Cheng, N. H., Folimonov, A., Hou, Y. M., Bao, Y. M., Katagi, C. *et al.* (2004). The Tobacco mosaic virus 126-kDa protein associated with virus replication and movement suppresses RNA silencing. *Mol Plant Microbe Interact* **17**: 583-592

10.������ Li, H., Li, W. X. & Ding, S. W. (2002). Induction and Suppression of RNA Silencing by an Animal Virus. *Science* **296**: 1319-1321

11.������ Thomas, C. L., Leh, V., Lederer, C. & Maule, A. J. (2003). Turnip crinkle virus coat protein mediates suppression of RNA silencing in Nicotiana benthamiana. *Virology* **306**: 33-41

12.������ Carrington, J. C., Kasschau, K. D. & Johansen, L. K. (2001). Activation and suppression of RNA silencing by plant viruses. *Virology* **281**: 1-5

13.������ Anandalakshmi, R., Pruss, G. J., Ge, X., Marathe, R., Smith, T. H. & Vance, V. B. (1998). A viral suppressor of gene silencing in plants. *Proc Natl Acad Sci USA* **95**: 13079-13084

14.������ Dunoyer, P., Pfeffer, S., Fritsch, C., Hemmer, O., Voinnet, O. & Richards, K. E. (2002). Identification, subcellular localization and some properties of a cysteine-rich suppressor of gene silencing encoded by peanut clump virus. *Plant J* **29**: 555-567

15.������ Pfeffer, S., Dunoyer, P., Heim, F., Richards, E. K., Jonard, G. & Ziegler-Graff, V. (2002). P0 of Beet Western Yellows Virus Is a Suppressor of Posttranscriptional Gene Silencing. *J Virol* **76**: 6815-6824

16.������ Bennasser, Y., Le, S. Y., Benkirane, M. & Jeang, K. T. (2005). Evidence that HIV-1 Encodes an siRNA and a Suppressor of RNA Silencing. *Immunity* **5**: 607-619

17.������ Marathe, R., Anandalakshmi, R., Smith, T. H., Pruss, G. J. & Vance, V. B. (2000). RNA viruses as inducers, suppressors and targets of post- transcriptional gene silencing. *Plant Mol Biol* **43**: 295-306

18.������ Vance, V. & Vaucheret, H. (2001). RNA silencing in plants--defense and counterdefense. *Science* **292**: 2277-2280

19.������ Dunoyer, P., Lecellier, C. H., Parizotto, E. A., Himber, C. & Voinnet, O. (2004). Probing the microRNA and small interfering RNA pathways with virus-encoded suppressors of RNA silencing. *Plant Cell* **16**: 1235-1250

20.������ Marathe, R., Smith, T. H., Anandalakshmi, R., Bowman, L. H., Fagard, M., Mourrain, P., Vaucheret, H. *et al.* (2000). Plant viral suppressors of post-transcriptional silencing do not suppress transcriptional silencing. *Plant J* **22**: 51-59

21.������ Mallory, A. C., Reinhart, B. J., Bartel, D., Vance, V. B. & Bowman, L. H. (2002). A viral suppressor of RNA silencing differentially regulates the accumulation of short interfering RNAs and microRNAs in tobacco. *Proc Natl Acad Sci USA* **99**: 15228-15233

22.������ Di Serio, F., Schob, H., Iglesias, A., Tarina, C., Bouldoires, E. & Meins, F. (2001). Sense- and antisense-mediated gene silencing in tobacco is inhibited by the same viral suppressors and is associated with accumulation of small RNAs. *Proc Natl Acad Sci USA* **98**: 6506-6510

23.������ Qu, F., Ren, T. & Morris, T. J. (2003). The coat protein of turnip crinkle virus suppresses posttranscriptional gene silencing at an early initiation step. *Journal of Virology* **77**: 511-522

24.������ Chen, J., Xiang, W. L., Xie, D., Peng, J. R. & Ding, S.-W. (2004). Viral Virulence Protein Suppresses RNA Silencing-Mediated Defense but Upregulates the Role of MicroRNA in Host Gene Expression. *Plant Cell* **16**: 1302-1313

25.������ Lakatos, L., Szittya, G., Silhavy, D. & Burgyan, J. (2004). Molecular mechanism of RNA silencing suppression mediated by p19 protein of tombsviruses. *EMBO J* **23**: 876-884

26.������ Llave, C., Kasschau, K. D. & Carrington, J. C. (2000). Virus-encoded suppressor of posttranscriptional gene silencing targets a maintenance step in the silencing pathway. *Proc Natl Acad Sci USA* **97**: 13401-13406

27.������ Lucy, A. P., Guo, H. S., Li, W. X. & Ding, S. W. (2000). Suppression of post-transcriptional gene silencing by a plant viral protein localized in the nucleus. *EMBO J* **19**: 1672-1680

28.������ Kasschau, K. D. & Carrington, J. C. (2001). Long-distance movement and replication maintenance functions correlate with silencing suppression activity of potyviral HC-Pro. *Virology* **285**: 71-81

29.������ Mallory, A. C., Ely, L., Smith, T. H., Marathe, R., Anandalakshmi, R., Fagard, M., Vaucheret, H. *et al.* (2001). HC-Pro suppression of transgene silencing eliminates the small RNAs but not transgene methylation or the mobile signal. *Plant Cell* **13**: 571-583

30.������ Trinks, D., Rajeswaran, R., Shivaprasad, P. V., Akbergenov, R., Oakeley, E. J., Veluthambi, K., Hohn, T. *et al.* (2005). Suppression of RNA Silencing by a Geminivirus Nuclear Protein, AC2, Correlates with Transactivation of Host Genes. *J Virol* **79**: 2517-2527

31.������ Baulcombe, D. C. (2002). Viral suppression of systemic silencing. *Trends Microbiol* **10**: 306-308

32.������ Kasschau, K. D., Xie, Z., Allen, E., Llave, C., Chapman, E. J., Krizan, K. A. & Carrington, J. C. (2003). P1/HC-Pro, a Viral Suppressor of RNA Silencing, Interferes with Arabidopsis Development and miRNA Function. *Dev Cell* **4**: 205-217

33.������ Hamilton, A. J., Voinnet, O., Chappell, L. & Baulcombe, D. C. (2002). Two classes of short interfering RNA in RNA silencing. *EMBO J* **21**: 4671-4679

34.������ Zamore, P. D. (2004). Plant RNAi: How a Viral Silencing Suppressor Inactivates siRNA. *Curr Biol* **14**: 3

35.������ Ye, K., Malinina, L. & Patel, D. J. (2003). Recognition of small interfering RNA by a viral suppressor of RNA Silencing. *Nature* **426**: 874-878

36.������ Silhavy, D. & Burgyan, J. (2004). Effects and side-effects of viral RNA silencing suppressors on short RNAs. *Trends Plant Sci* **9**: 76-83

37.������ Qi, Y., Zhong, X., Itaya, A. & Ding, B. (2004). Dissecting RNA silencing in protoplasts uncovers novel effects of viral suppressors on the silencing pathway at the cellular level. *Nucleic Acids Res* **32**: e179

38.������ Chapman, E. J., Prokhnevsky, A. I., Gopinath, K., Dolja, V. & Carrington, J. C. (2004). Viral RNA silencing suppressors inhibit the microRNA pathway at an intermediate step. *Genes Dev* **18**: 1179-86

39.������ Anandalakshmi, R., Marathe, R., Ge, X., Herr Jr., J. M., Mau, C., Mallory, A., Pruss, G. *et al.* (2000). A Calmodulin-Related Protein That Suppresses Posttranscriptional Gene Silencing in Plants. *Science* **290**: 142-144

Endogenous siRNAs

Endogenous siRNAs are small RNAs that are encoded by an organism�s own genome but are not miRNAs. The term �endogenous small RNAs� can be used to collectively refer to miRNAs and endogenous siRNAs. Following the discovery of siRNAs in 1999, two laboratories demonstrated that there are siRNA in plant in which RNA silencing had not been triggered by a transgene or a virus. These siRNAs were shown to originate from regions between protein-coding genes, from repetitive DNA sequences and from transposons [1,2] . Transposons are mobile genetic elements that are akin to some viruses and transposon-derived siRNAs play an important role in restricting the activity of these elements that could otherwise cause extensive mutations [3,4,5] . Many more endogenous siRNAs have been reported since then [6,7,8] .

While transposon-derived endogenous siRNAs only affect the transposon they are derived from, �trans-acting siRNAs� regulate other genes in a miRNA-like manner [7,9] .

Endogenous siRNAs differ from miRNAs in the way they are produced. A miRNA gene is transcribed to produce a folded, partially double-stranded RNA, the miRNA-precursor, from which a precisely defined miRNA is excised [10] (see Figure). In contrast, other regions within the genome can give rise to extended double-stranded RNAs that are processed by Dicer enzymes to form a diverse population of more or less overlapping endogenous siRNAs.

Endogenous siRNAs that are derived from repeated DNA elements and transposons require the activity of DNA-dependent RNA-polymerase 4 (also known as SDE4), Dicer-Like3 (DCL3) and RNA-dependent RNA-polymerase 2 (RDR2) for their biosynthesis [2,11,12] . In contrast, trans-acting siRNAs, a subgroup of endogenous siRNAs, are produced in a process that involves RNA-dependent RNA-polymerase 6 (RDR6) but not DCL3 or RDR2 [7] . In addition, the production of trans-acting siRNAs is linked to the miRNA pathway [7] .

Literature

1.�������� Llave, C., Kasschau, K. D., Rector, M. A. & Carrington, J. C. (2002). Endogenous and silencing-associated small RNAs in plants. *Plant Cell* **14**: 1605-1619

2.�������� Hamilton, A. J., Voinnet, O., Chappell, L. & Baulcombe, D. C. (2002). Two classes of short interfering RNA in RNA silencing. *EMBO J* **21**: 4671-4679

3.�������� Waterhouse, P. M., Wang, M. B. & Lough, T. (2001). Gene silencing as an adaptive defence against viruses. *Nature* **411**: 834-842

4.�������� Aravin, A. A., Lagos-Quintana, M., Yalcin, A., Zavolan, M., Marks, D., Snyder, B., Gaasterland, T. *et al.* (2003). The small RNA profile during Drosophila melanogaster development. *Dev Cell* **5**: 337-350

5.�������� Sunkar, R., Girke, T. & Zhu, J.-K. (2005). Identification and characterization of endogenous small interfering RNAs from rice. *Nucleic Acids Res* **33**: 4443-4454

6.�������� Sunkar, R. & Zhu, J.-K. (2004). Novel and Stress-Regulated MicroRNAs and Other Small RNAs from Arabidopsis. *Plant Cell* **16**: 2001-2019

7.�������� Vazquez, F., Vaucheret, H., Rajagopalan, R., Lepers, C., Gasciolli, V., Mallory, A. C., Hilbert, J.-L. *et al.* (2004). Endogenous trans-acting siRNAs Regulate the Accumulation of Arabidopsis mRNAs. *Mol Cell* **16**: 69-79

8.�������� Gustafson, A. M., Allen, E., Givan, S., Smith, D., Carrington, J. C. & Kasschau, K. D. (2005). ASRP: the Arabidopsis Small RNA Project Database. *Nucleic Acids Res* **33**: D637-D640

9.�������� Peragine, A., Yoshikawa, M., Wu, G., Albrecht, H. L. & Poething, R. S. (2004). *SGS3* and *SGS2/SDE1/RDR6* are required for juvenile development and the production of *trans-acting* siRNAs in *Arabidopsis*. *Genes Dev* **18**: 2368-2379

10.������ Bartel, D. P. (2004). MicroRNAs: Genomics, Biogenesis, Mechanism, and Function. *Cell* **116**: 281-297

11.������ Xie, Z., Johansen, L. K., Gustafson, A. M., Kasschau, K. D., Lellis, A. D., Zilberman, D., Jacobsen, S. E. *et al.* (2004). Genetic and Functional Diversification of Small RNA Pathways in Plants. *PLoS Biology* **2**: 642-652

12.������ Herr, A. J., Jensen, M. B., Dalmay, T. & Baulcombe, D. (2005). RNA polymerase IV directs silencing of endogenous DNA. *Science* **308**: 118-120

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Transposable elements, (transposons)

Transposable elements are DNA sequences with the ability to move from place to place within a genome. They are divided into two classes. Class 1 transposable elements (retroelements) make up over 70% of genomic DNA in maize. Retroelements multiply via an RNA intermediate (a process called reverse transcription). Whereas a copy of a class 1 element remains at its original location during transposition, class 2 transposable elements excise themselves from one location to integrate into a new place in the genome.
Many endogenous siRNAs in animals and plants are derived from transposons, showing that silencing these otherwise mutagenic elements is an important part of genome maintenance [1,2,3] .

Literature

1.�������� Aravin, A. A., Lagos-Quintana, M., Yalcin, A., Zavolan, M., Marks, D., Snyder, B., Gaasterland, T. *et al.* (2003). The small RNA profile during Drosophila melanogaster development. *Dev Cell* **5**: 337-350

2.�������� Sunkar, R., Girke, T. & Zhu, J.-K. (2005). Identification and characterization of endogenous small interfering RNAs from rice. *Nucleic Acids Res* **33**: 4443-4454

3.�������� Waterhouse, P. M., Wang, M. B. & Lough, T. (2001). Gene silencing as an adaptive defence against viruses. *Nature* **411**: 834-842

Trans-acting (ta) siRNAs

Trans-acting (ta-) siRNAs are a class of endogenous siRNAs. Acting in �trans� means that the targets of these siRNAs are different from the transcripts that give rise to them. Similar to miRNAs, ta-siRNAs are generated from precursor RNAs that are encoded by the genome [1,2] . Clusters of ta-siRNAs occur in the genome because each precursor transcript is processed into several non-overlapping siRNAs [1,2,3] .
MiRNAs play a role in the biosynthesis of ta-siRNAs by introducing a cleavage in the precursor RNA. This seems to be recognised by RNA-dependent RNA polymerase 6 (RDR6), which converts the single-stranded precursor into the double-stranded form. A Dicer enzyme (DCL4) process the doubles-stranded precursor into the mature ta-siRNAs [1,4] . Another Dicer, DCL1, is required for miRNA maturation and therefore also for ta-siRNA biosynthesis [5,6] .

Literature

1.�������� Peragine, A., Yoshikawa, M., Wu, G., Albrecht, H. L. & Poething, R. S. (2004). *SGS3* and *SGS2/SDE1/RDR6* are required for juvenile development and the production of *trans-acting* siRNAs in *Arabidopsis*. *Genes Dev* **18**: 2368-2379

2.�������� Vazquez, F., Vaucheret, H., Rajagopalan, R., Lepers, C., Gasciolli, V., Mallory, A. C., Hilbert, J.-L. *et al.* (2004). Endogenous trans-acting siRNAs Regulate the Accumulation of Arabidopsis mRNAs. *Mol Cell* **16**: 69-79

3.�������� Reinhart, B. J., Weinstein, E. G., Rhoades, M., Bartel, B. & Bartel, D. P. (2002). MicroRNAs in plants. *Genes Dev* **16**: 1616-1626

4.�������� Allen, E., Xie, Z., Gustafson, A. M. & Carrington, J. C. (2005). microRNA-Directed Phasing during Trans-Acting siRNA Biogenesis in Plants. *Cell* **121**: 207-221

5.�������� Xie, Z., Allen, E., Wilken, A. & Carrington, J. C. (2005). DICER-LIKE 4 functions in trans-acting small interfering RNA biogenesis and vegetative phase change in Arabidopsis thaliana. *Proc Natl Acad Sci USA* **102**: 12984-12989

6.�������� Yoshikawa, M., Peragine, A., Park, M. Y. & Poethig, R. S. (2005). A pathway for the biogenesis of trans-acting siRNAs in Arabidopsis. *Genes Dev* **19**: 2164-2175

Bbiosynthesis

The term biosynthesis refers to a production process *in-vitro* by which simple precursors are processed by enzymes into more complex compounds.

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CG, CNG and CNN methylation

DNA methylation affects cytosine (C) nucleotides and is linked to RNA silencing. There are two phases of DNA methylation: initiation and maintenance. Initiation requires a trigger which can be a double-stranded RNA. When DNA is replicated, the methylation pattern is copied onto the newly synthesised strands to maintain the methylation status. In plants, DNA methylation is preferably maintained at cytosines in the CG context (which refers to a cytosine that is followed by a guanine) and in CNG contexts (where N can be any nucleotide other than G). Cytosines in other sequence contexts can also be methylated but this type of methylation is not maintained in absence of the original trigger.

Initiation and maintenance of methylation patterns in plants involves at least three different types of methyltransferases, i.e. enzymes that add methyl groups. DRM methyltransferases can initiate new methylation but are not involved in maintaining pre-established methylation patterns [1,2,3,4] . In contrast, the CMT3 (for CNG contexts) and MET1 (for CG contexts) methyltransferases are required for the maintenance of methylation patterns in the absence of a trigger but not for the initial establishment of these patterns [2,4,5,6,7,8] .

DNA methylation is linked to heterochromatinisation, a process that changes the packing density of regions within the genome. Densely packed heterochromatin can attract CMT3 methylatransferases and guide them to at least some DNA regions where CNG methylation needs to be maintained [9,10] .

Literature

1.��������� Wada, Y., Ohya, H., Yamaguchi, Y., Koizumi, N. & Sano, H. (2003). Preferential de Novo Methylation of Cytosine Residues in Non-CpG Sequences by a Domains Rearranged DNA Methyltransferase from Tobacco Plants. *J Biol Chem* **278**: 42386-42393

2.��������� Cao, X. & Jacobsen, S. E. (2002). Locus-specific control of asymmetric and CpNpG methylation by the DRM and CMT3 methyltransferase genes. *Proc Natl Acad Sci USA* **99**: 16491-16498

3.��������� Cao, X. F. & Jacobsen, S. E. (2002). Role of the Arabidopsis DRM methyltransferases in de novo DNA methylation and gene silencing. *Curr Biol* **12**: 1138-1144

4.��������� Cao, X., Aufsatz, W., Zilberman, D., Mette, M. F., Huang, M. S., Matzke, M. & Jacobsen, S. E. (2003). Role of the *DRM* and *CMT3* Methyltransferases in RNA-Directed DNA Methylation. *Curr Biol* **13**: 2212-2217

5.��������� Lindroth, A. M., Cao, X. F., Jackson, J. P., Zilberman, D., McCallum, C. M., Henikoff, S. & Jacobsen, S. E. (2001). Requirement of CHROMOMETHYLASE3 for maintenance of CpXpG methylation. *Science* **292**: 2077-2080

6.��������� Bartee, L., Malagnac, F. & Bender, J. (2001). *Arabidopsis cmt3* chromomethylase mutations block non-CG methylation and silencing of an endogenous gene. *Genes Dev* **15**: 1753-1758

7.��������� Kankel, M. W., Ramsey, D. E., Stokes, T. L., Flowers, S. K., Haag, J. R., Jeddeloh, J. A., Riddle, N. C. *et al.* (2003). Arabidopsis MET1 cytosine methyltransferase mutants. *Genetics* **163**: 1109-1122

8.��������� Saze, H., Scheid, O. M. & Paszkowski, J. (2003). Maintenance of CpG methylation is essential for epigenetic inheritance during plant gametogenesis. *Nature Genetics* **34**: 65-69

9.��������� Jackson, J. P., Lindroth, A. M., Cao, X. F. & Jacobsen, S. E. (2002). Control of CpNpG DNA methylation by the KRYPTONITE histone H3 methyltransferase. *Nature* **416**: 556-560

10.������� Malagnac, F., Bartee, L. & J., B. (2002). An Arabidopsis SET domain protein required for maintenance but not establishment of DNA methylation. *EMBO J* **21**: 6842-6852

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Chromatin

Literally meaning �stainable matter� this term refers to the genomic DNA and its associated packaging proteins in the nucleus of a cell. DNA is wrapped around a protein �reel� composed of histones H2A, H2B, H3 and H4. A �nucleosome� is a unit consisting of the histone core and about 150 basepairs of DNA wrapped around it. Nucleosomes can be more or less condensed and the degree of condensation affects the accessibility and, hence, the transcriptional activity of the region. In general, a higher degree of condensation reduces the activity of a genomic region but there are exceptions to this rule. Heterochromatin is the term used for a densely packed region. The opposite is euchromatin. The pattern of more or less densely packed DNA that becomes visible when chromosomes are condensed during cell-division has been known since the 1920s [1] .

Literature

1.�������� Heitz, E. (1928). [Das Heterochromatin der Moose]. *Jahrbuch der Wissenschafltichen Botanik* **69**: 762-818

Heterochromatin

Densely packed chromatin is referred to as heterochromatin. In general, DNA in heterochromatic regions is less accessible to the transcription machinery than DNA in euchromatic regions. As a result, genes in heterochromatic regions are expressed at low levels or they are completely silenced. However, heterochromatin does not always inactivate genes and some genes even require a heterochromatic environment to be active [1]
DNA is wrapped around structural proteins (histones) that can be chemically modified to affect the chromatin status. The condensation of euchromatin to heterochromatin is called heterochromatinisation.

Literature

1.�������� Eissenberg, J. C. & Wallrath, L. L. (2003). Heterochromatin, Position Effects, and the Genetic Dissection of Chromatin. *Prog Nucleic Acid Res Mol Biol* **74**: 275-299

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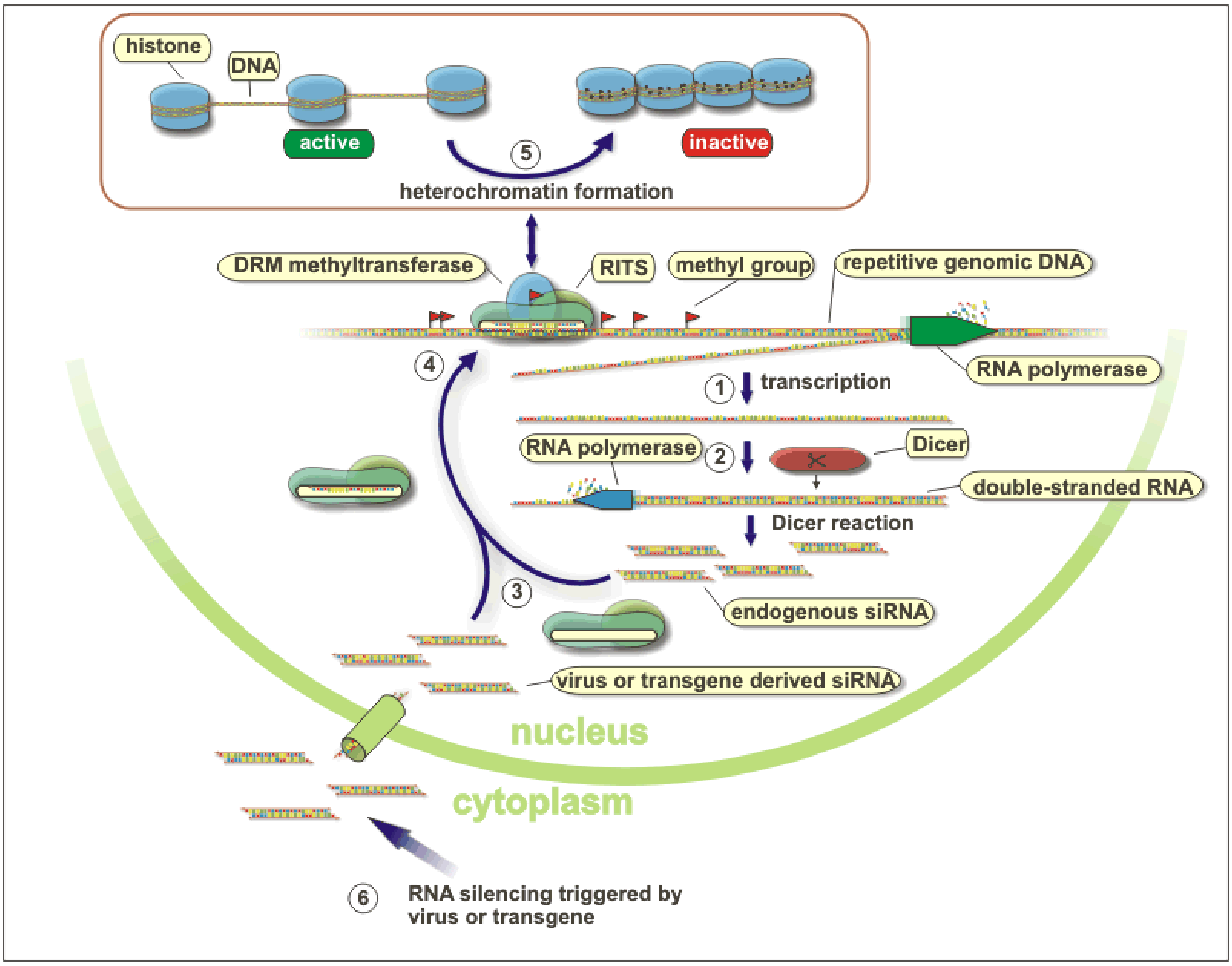
RNA-induced methylation and chromatin modification



This illustration gives an overview of RNA-induced DNA methylation and chromatin modification pathways

Genomic regions that contain repetitive sequence elements are transcribed into RNA by a specialised RNA-polymerase (1). These transcripts are recognised by another RNA-polymerase and converted into a double-stranded RNA (2), which is processed by Dicer. The products of the Dicer reaction are endogenous siRNAs (derived from the plant's own genome). As in post-transcriptional silencing pathways, the siRNAs are incorporated into an effector complex (3), referred to as RITS (RNA-induced transcriptional gene silencing complex). The siRNA guides the complex to matching regions within the genomic DNA (4) where it induces a chemical modification that does not alter the DNA sequence. This reaction involves a DRM methyltransferease, an enzyme that adds methyl groups to cytosine residues. A different set of methyltransferases is required to maintain the methylation pattern during DNA replication.

Methylated DNA is less accessible to components of the transcription machinery. Furthermore, DNA methylation is linked to heterochromatin formation: DNA is wrapped around structural proteins, termed histones. Changing the structure from loosely packed "euchromatin" to densely packed "heterochromatin" inactivates genes within the affected region (5). Heterochromatin, in turn, attracts the RNA-polymerase that produces the templates for endogenous siRNAs, thus enabling a self-sustaining feed-back loop to maintain the silenced state.

SiRNAs generated from viral RNAs or transgenes can also feed into the methylation/chromatin-modification pathway (6).

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Methylation

A methyl group consists of one carbon and three hydrogen atoms. Methyl groups are used by many organisms to modify DNA, RNA or proteins. The process of adding a methyl group is known as �methylation�.
The methyl group often functions as a marker that attracts other proteins for further modifications. DNA methylation of promoter sequences (elements that control the expression of the adjacent genes) leads to�transcriptional silencing of the target gene [1] . There are different types of DNA-methylation for different sequence contexts. These are known as CG, CNG and CNN methylation.
Once established, DNA methylation can be maintained in the absence of the original trigger and, in plants, the methylation pattern is inherited by the progeny [2] . When DNA is replicated one strand carries the imprinted methylation pattern while the newly synthesised strand does not. The methylation maintenance machinery of the cell recognises such hemi-(half-)methylated DNA and imprints the methylation pattern onto the newly synthesised strand. This methylation pattern is rarely actively deleted. It can be lost, however, as a consequence of a failure in the maintenance process. [3] .
The maintenance of methylation patterns in plant genomes depends on the activity of methyltransferases such as MET1 or CMT3 and also requires the DDM1 chromatin remodelling helicase [4] .
Small RNAs such as siRNAs and miRNAs can also be methylated. In plants, the HEN1 protein methylates miRNAs [5] . In this case the methylation occurs at the ribose backbone and not at a nucleobase. In addition to providing a quality control step in their biosynthesis, methylation might be required to prevent miRNAs from acting as primers for RNA-polymerases, which could cause undesirable transitive silencing on endogenous target genes. Methylated miRNAs might also be more stable than non-methylated ones. Similarly, methylation has been shown to increase the stability of artificial siRNAs in blood, an essential prerequisite for applications of RNA silencing in medicine [6] .

Literature

1.�������� Matzke, A. J. & Birchler, J. A. (2005). RNAi-mediated pathways in the nucleus. *Nat Rev Genet* **6**: 24-35

2.�������� Jones, L., Ratcliff, F. & Baulcombe, D. C. (2001). RNA-directed transcriptional gene silencing in plants can be inherited independently of the RNA trigger and requires Met1 for maintenance. *Curr Biol* **11**: 747-757

3.�������� Bender, J. (2004). DNA Methylation and Epigenetics. *Annu Rev Plant� Biol* **55**: 41-68

4.�������� Jeddeloh, J. A., Bender, J. & Richards, E. J. (1998). The DNA methylation locus DDM1 is required for maintenance of gene silencing in Arabidopsis. *Genes Dev* **12**: 1714-1725

5.�������� Yu, B., Yang, Z., Li, J., Minakhina, S., Yang, M., Padgett, R. W., Steward, R. *et al.* (2005). Methylation as a Crucial Step in Plant microRNA Biogenesis. *Science* **307**: 932-935

6.�������� Soutschek, J., Akinc, A., Bramlage, B., Charisse, K., Constien, R., Donoghue, M., Elbashir, S. M. *et al.* (2004). Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature* **432**: 173-178

Epigenetic modifications

Epigenetic modifications are minor chemical modifications of nucleobases and DNA packaging proteins which affect the expression pattern of a gene without changing its sequence. Transcriptional silencing of a gene as a result of RNA-induced DNA methylation is an example for an epigenetic modification.

DRM methyltransferases (*Arabidopsis*)

DRM methyltransferases can initiate methylation at CG, CNG and CNN sites but are not involved in maintaining pre-established methylation patterns [1,2,3,4]

Literature

1.�������� Wada, Y., Ohya, H., Yamaguchi, Y., Koizumi, N. & Sano, H. (2003). Preferential de Novo Methylation of Cytosine Residues in Non-CpG Sequences by a Domains Rearranged DNA Methyltransferase from Tobacco Plants. *J Biol Chem* **278**: 42386-42393

2.�������� Cao, X. & Jacobsen, S. E. (2002). Locus-specific control of asymmetric and CpNpG methylation by the DRM and CMT3 methyltransferase genes. *Proc Natl Acad Sci USA* **99**: 16491-16498

3.�������� Cao, X. F. & Jacobsen, S. E. (2002). Role of the Arabidopsis DRM methyltransferases in de novo DNA methylation and gene silencing. *Curr Biol* **12**: 1138-1144

4.�������� Cao, X., Aufsatz, W., Zilberman, D., Mette, M. F., Huang, M. S., Matzke, M. & Jacobsen, S. E. (2003). Role of the *DRM* and *CMT3* Methyltransferases in RNA-Directed DNA Methylation. *Curr Biol* **13**: 2212-2217

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MET1

In plants, the methyltransferase MET1 is required for the maintenance and inheritability but not the establishment of DNA CG methylation patterns that are associated with transcriptional gene silencing [1,2] . In contrast, maintenance of DNA methylation associated with post-transcriptional gene silencing is MET1-independent [2] . Although MET1 has a role in seed development and flowering, mutations in MET1 are not lethal to plants.

Literature

1.�������� Finnegan, E. J. & Kovac, K. A. (2000). Plant DNA methyltransferases. *Plant Mol Biol* **43**: 189-201

2.�������� Jones, L., Ratcliff, F. & Baulcombe, D. C. (2001). RNA-directed transcriptional gene silencing in plants can be inherited independently of the RNA trigger and requires Met1 for maintenance. *Curr Biol* **11**: 747-757

RNA-induced initiation of transcriptional gene silencing complex (RITS complex)

While RISC is the effector complex of post-transcriptional gene silencing, RITS is the effector complex of transcriptional gene silencing. Its existence has been shown in fission yeast, where it contains an ARGONAUTE protein, the chromodomain protein Chp1, Tas3 (a protein of unknown function) and Dicer-generated siRNAs [1]. RITS localises to all heterochromatic regions in fission yeast where it is involved in a self-enforcing loop mechanism [2]: RITS is tethered to the methylated histones in the heterochromatic target region, probably by the chromodomain protein Chp1 [3]. RITS promotes the processing of RNA that is transcribed from the region it is bound to. This processing involves an RNA-dependent RNA-polymerase and results in the formation of double-stranded RNA that is processed into siRNAs by Dicer [4,5]. These siRNAs target the region they are derived from to maintain the heterochromatic state and promote binding of RITS [1]. See Figure.

Literature

1.�������� Verdel, A., Jia, S., Gerber, S., Sugiyama, T., Gygi, S., Grewal, S. I. S. & Moazed, D. (2004). RNAi-Mediated Targeting of Heterochromatin by the RITS Complex. *Science* **303**: 672-676

2.�������� Noma, K., Sugiyama, T., Cam, H., Verdel, A., Zofall, M., Jia, S., Moazed, D. *et al.* (2004). RITS acts in cis to promote RNA interference-mediated transcriptional and post-transcriptional silencing. *Nat Genet* **36**: 1174-1180

3.�������� Partridge, J. F., Scott, K. S. C., Bannister, A. J., Kouzarides, T. & Allshire, R. C. (2002). cis-Acting DNA from Fission Yeast Centromeres Mediates Histone H3 Methylation and Recruitment of Silencing Factors and Cohesin to an Ectopic Site. *Curr Biol* **12**: 1652-1660

4.�������� Sugiyama, T., Cam, H., Verdel, A., Moazed, D. & Grewal, S. I. S. (2005). RNA-dependent RNA polymerase is an essential component of a self-enforcing loop coupling heterochromatin assembly to siRNA production. *Proc Natl Acad Sci USA* **102**: 152-157

5.�������� Motamedi, M. R., Verdel, A., Colmenares, S. U., Gerber, S. A., Gygi, S. P. & Moazed, D. (2004). Two RNAi Complexes, RITS and RDRC, Physically Interact and Localize to Noncoding Centromeric RNAs. *Cell* **119**: 789-802

Table 1 � Applications of RNA silencing in GM crops

Crop	Silencing technology used	Target	Purpose and comments	Reference
Apple	sense-suppression	DspE-interacting kinases	The plant-pathogenic bacterium *Erwinia amylovora* secrets the DspE protein. To establish the disease, this protein must interact with a group of plant proteins, the DspE-interacting kinases. Silencing the latter therefore prevents the disease.	313
Coffee	double-stranded RNA	Theobromine synthase 1 (MXMT1)	Reducing the caffeine content in coffee plants. In this case, targeting theobromine synthase 1 also led to down-regulation of two other genes which are involved in caffeine synthesis. The resulting coffee plants exhibited reduced caffeine levels.	181
Cotton	double-stranded RNA and antisense suppression	DELTA9-desaturase, oleoyl-phosphatidylcholine omega6-desaturase, delta-cadinene synthase	Improving the fatty-acid composition of cotton seed oil.	204,314
Diverse plant species	sense and antisense suppression, double-stranded RNA	plant viruses	RNA silencing is a natural defence mechanism of plants against viruses. This can be exploited to pre-establish an �immunised� state	315,316,317,8,10,11,257,278,316,318,319, 320,321,322, 323

			against economically important viruses.� This is the most extensively examined application of RNA silencing in transgenic plants. The second GM crop to be released for commercial use was a virus-resistant squash. Although this plant, as well as many other in the literature, had not been designed to employ RNA silencing against the virus, we now know that this is the mechanism behind pathogen-derived resistance in most of the cases reported in the literature.	
Maize	antisense suppression	O-methyltransferase	The GM plants exhibit reduced lignin contents, which facilitates digestion of this forage grass in animals, thus improving livestock performance. This could be particularly useful in tropical forage species, which generally are of lower quality than species from temperate climates.	324
Maize	double-stranded RNA	22-kD zein	Zein is a storage proteins in maize seeds. Suppressing zeins improves the nutritional value of maize.	102

Opium poppy	double-stranded RNA	codeinone reductase (COR)	Replacement of morphine with a nonnarcotic metabolite.	325
Ornamental crops	antisense suppression and double-stranded RNA	Enzymes involved in flower pigmentation	Manipulating flower colours in ornamental crops.	2,3,180,276, 326
Poplar	antisense suppression	cinnamyl-alcohol dehydrogenase (CAD) or caffeate/5-hydroxy-ferulate O-methyltransferase	Improving pulping characteristics for papermaking.	281
Potato	antisense suppression	Plastidic glutamine synthase	The target enzyme is involved in photorespiration, a process that competes with photosynthesis and is triggered by high oxygen levels. It is normally avoided by the plant but suppression of plastidic glutamine synthase forces the plant metabolism into photorespiration mode, unless a high carbon monoxide pressure is provided. As this is only possible in a controlled environment, these plants can not survive outside the greenhouse. This construct would be used as an addition in transgenic plants that might pose a health and safety risk in the environment, e.g.	207

			transgenic plants that produce pharmacological substances.	
Potato	antisense suppression	G1-1 and A2-1	Increased dormancy periods of tubers to prevent germinating during storage.	327
Potato	antisense suppression	Threonine synthase	Changing the aminoacid metabolism of the plant to improve nutritional value. In this case, two braches of a pathway use the same precursor substance to produce two different amino acids. Suppressing one branch therefore leads to increased channelling of the precursor into the remaining branch.	328
Potato	sense-suppression and antisense suppression	Granule-bound starch synthase I (GBSSI)	Silencing GBSSI leads to reduced amylose contents in tubers.	329
Rice	antisense suppression	allergenic proteins	Reducing the accumulation of allergenic proteins.	330,331
Rice	antisense suppression	Waxy	The protein Waxy is involved in amylose metabolism. The resulting GM plants exhibit lower amylose levels in the seeds, which has a positive effect on the processing characteristics of rice.	332

Ryegrass	antisense suppression	Lol p 5	Lol p 5 is the major allergenic protein of ryegrass pollen. In this study, it was targeted by pollen-specific expression of Lol p 5 antisense suppression RNA, resulting in reduced allergenicity.	333
Soybean	antisense suppression	allergenic proteins	Reducing the accumulation of allergenic proteins.	184
Sweet potato	sense-suppression	Granule-bound starch synthase I (GBSSI)	Silencing GBSSI leads to reduced amylose contents in tubers.	334
Tobacco	antisense suppression	any transgene	The antisense suppression construct is expressed under a pollen-specific promoter, i.e. the silencing trigger is only present in pollen, where it suppresses the production of the targeted transgene-product. This system might be useful to prevent uncontrolled spreading of a protein from transgenic plants via pollen. Tobacco is only used as a model plant in this study.	206
Tobacco	double-stranded RNA	Influenza NS1 protein	In this case, the plant is engineered to express siRNAs targeting a human pathogen - the influenza virus. In this experiment, the RNA, including the virus-specific siRNA, was	134

			harvested from the plant and introduced into human cells, which successfully inhibited viral replication.	
Tomato	double-stranded RNA	polygalacturonase	This is the Flavr Svr� �Tomato, which exhibits delayed fruit softening. It has been shown that the target is silenced due to aberrant integration of the transgene into the genome, leading to the expression of double-stranded RNA rather than the expected antisense trigger.	110,111
Tomato	antisense suppression	ACC synthase	Suppression of components of the ethylene metabolism reduces the susceptibility of the plant to a herbicide.	335
Tomato	double-stranded RNA	DE-ETIOLATED1 (DET1)	DET1 is a regulatory gene involved in several signalling pathways controlled by light. Silencing DET1 therefore influences many metabolic pathways, which has a detrimental effect on growth and development of the plant. In this study, DET1 silencing is triggered in fruits only, using a fruit-specific promoter. As a consequence, the plants grow normally	202

			but the fruits accumulate increased levels of lycopene and β-carotene, which are highly beneficial to human health.	
Walnut	double-stranded RNA	tryptophan monooxygenase (iaaM) and isopentenyltransferase (ipt) from Agrobacterium tumefaciens.	Agrobacterium tumefaciens is a bacterial pathogen that infects many plant species, leading to crown gall disease. The bacterium inserts parts of its own genome into the plant genome, thus forcing the plant to produce the nutrients it requires. Silencing these bacterial genes in the plant prevents the disease.	336

in-vitro, in-vivo

In-vitro means �in the glass�, i.e. in the test tube, as opposed to *in-vivo* studies that analyse reactions in a living cell/organism.

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Off-target silencing

For the purpose of this report, off-target silencing is defined as a silencing effect on a non-target gene that was neither intended nor predicted. Off-target effects are observed because we do not know all the rules that govern the interaction between a silencing trigger and its target or because the target organism is not fully sequenced.

Is is important to distinguish off-target 'primary' effects, i.e. silencing of a non-target RNA by a direct interaction between trigger and target, and 'secondary' effects, i.e. effects caused by the specific down-regulation of the target. Many genes are part of complex egulation networks so that down-regulating one gene can influence the expression of other genes. Secondary effects can also result from transgenic over-expression strategies. In addition, there may be non-specific effects on non-target genes caused by flooding the cell with triggers of RNA silencing.

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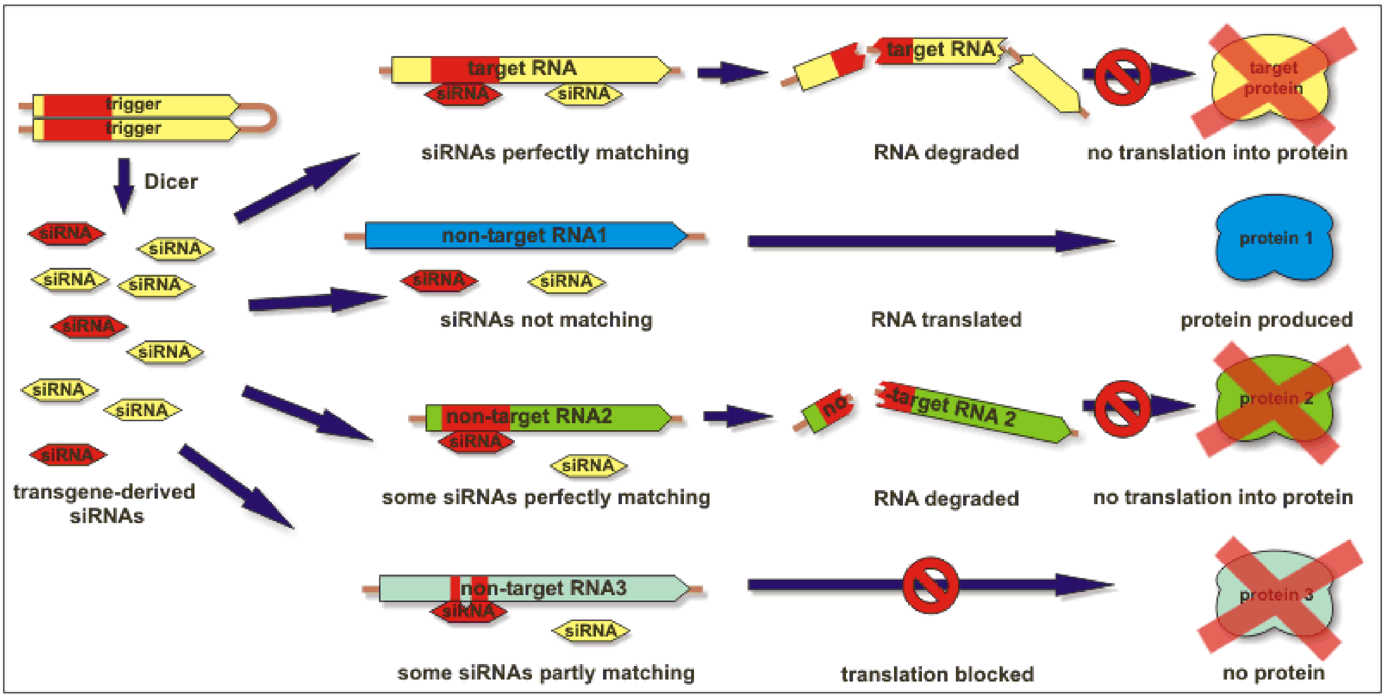
Hypothetical hazards: off-target effects (2 slides)



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Off-target effects

The sequence-specificity of RNA silencing is mediated by small interfering (si)RNAs. In the example shown here, the siRNAs are generated by an inverted-repeat RNA (see here for details) that is identical in sequence to the target messenger RNA (top).
The three messenger RNAs below represent three different types of possible interaction between transgene-derived siRNAs and non-target RNAs:

- Non-target RNA 1 does not share any sequence with the target RNA and is therefore not affected.
- Non-target RNA 2 has a region of identical sequence in common with the target RNA (shown in red). This RNA is targeted for degradation by siRNAs derived from the common region.
- Non-target RNA 3 has at least one short fragment of sequence in common with the target gene. This fragment may be shorter than an siRNA. In some cases, if there are only a few mismatches between siRNA and target, this may still induce target degradation. If there are too many mismatches the siRNA can not induce degradation of the target RNA but it might block translation instead. The abundance of the protein product, but not the messenger RNA, of the non-target gene is affected in this case.

Effects such as those shown here for RNAs 2 and 3 are predictable to a certain degree if sufficient sequence information is available and in some cases they are induced deliberately to silence several members of a gene family at once.

Silencing a gene can also induce secondary effects on non-target genes.

Microarray assay

Rather than monitoring expression levels of a single gene in different tissues and under different environmental conditions, researchers often want to analyse patterns of gene expression. A DNA microarray contains thousands of DNA probes, densely spotted onto a chip, which enables sequence-specific genome-wide quantification of messenger RNAs. One sample is taken as a reference (control) before the experiment is started. The experimental dataset is then computationally compared to the control dataset to reveal the impact of the experimental conditions on the messenger RNA expression pattern. Experiments like these have been used to investigate regulatory effects of siRNAs on non-target messenger RNAs. Microarrays are now also being developed to examine the expression patterns of known miRNAs [1] .

Literature

1.��������� Shingara, J., Keiger, K., Shelton, J., Laosinchai-Wolf, W., Powers, P., Conrad, R., Brown, D. *et al.* (2005). An optimized isolation and labeling platform for accurate microRNA expression profiling. *RNA* **11**: 1461-1470

Table 2 � overview of sequence requirements for the siRNA-target interaction.

A) Experiments analysing the effect of mismatches on target messenger RNA silencing

Organism	Experiment	Number and position of mismatches in siRNA* → effect on silencing				Comments	References
		all regions	3� end	centre	5�end		
Isolated mammalian cells	Synthetic siRNA, predicted target monitored			up to 4 → mode of action changes from cleavage to translational repression			68,149,183
Insect� embryo extract	Synthetic siRNA, protein expression of predicted target monitored		4 → no silencing	4 → no silencing 1 at cleavage site → no silencing	2 or 4 → very low level of silencing		137
Plant	Short trigger integrated in virus (27 nucleotides), protein expression of predicted target monitored			1 → no silencing			36
Insect embryos	Synthetic siRNA, phenotype monitored			1 → mild effect on silencing 2 → significantly reduced silencing efficacy		Silencing was evaluated by scoring effects on embryo development but target expression levels were not quantified	337
Isolated mammalian cells	Synthetic siRNA, mRNA and protein levels of predicted			1 → very mild effect on silencing 2 → reduced silencing efficacy but mRNA still		The effect of 2 central mismatches is difficult to explain. The endogenous target mRNA level was significantly reduced but in another assay, almost	152

	target monitored			significantly reduced. No clear effect in translational assay		no effect of the mismatched siRNA on target translation was observed.	
Isolated mammalian cells	Synthetic siRNA, replication of predicted target (a virus) monitored			1 → differential effect depending on target site, one siRNA had almost full silencing efficacy while another one was significantly less effective 4 → no silencing			151
Isolated mammalian cells	Synthetic siRNA, replication of predicted target (a virus) monitored			1 → silencing abolished or at least significantly reduced	1 → silencing abolished or at least significantly reduced	The synthetic siRNA had no mismatches to the viral RNA target but after long incubation times, a mutated virus with one mismatch to the siRNA at a central position appeared.	153,287
Isolated mammalian cells	Synthetic siRNA, replication of predicted target (a virus) monitored			1 → silencing abolished or at least significantly reduced		Similar to the findings of Gitlin *et al.* [287], the HIV virus escaped from being targeted by an shRNA silencing trigger construct after acquiring a mutation in the central region of the shRNA target site.	338
Plant	Synthetic siRNAs, protein expression of predicted target monitored	6 → no silencing					45
Isolated mammalian cells	Synthetic siRNA, mRNA and protein			2 → no silencing			339,340

	levels of predicted target monitored						
Isolated mammalian cells	Synthetic siRNA and shRNA, mRNA and protein levels of predicted target monitored			1 → significantly reduced silencing efficacy 2 → no silencing			341
Isolated mammalian cells	Synthetic siRNA, mRNA and protein levels of target monitored	3 G-U wobble mismatches → significantly reduced silencing efficacy		1 G-U wobble mismatch → significantly reduced silencing efficacy	1 G-U wobble mismatch → no effect 1 true mismatch → significantly reduced silencing efficacy		148
Isolated mammalian cells	Synthetic siRNA, mRNA and protein levels of target monitored			2 G-U wobble mismatches and 3 true mismatches → mode of action changes from cleavage to translational repression			149
Isolated mammalian cells	Synthetic siRNA, mRNA and protein levels of target monitored		1 in position 1 or 2 → no effect 1 in positions 5-11 → significantly	1 in positions 5-11 → significantly reduced or completely abolished silencing	1 in positions 12-17 → reduced silencing efficacy.	The effect of mutations depended not only on the position but on the identity of the substituted nucleobase to the extent that some mutations in regions of otherwise low tolerance were well tolerated.	147

			reduced or completely abolished silencing				
Isolated mammalian cells	Synthetic siRNA, mRNA levels of Predicted target monitored		1 → significantly reduced silencing efficacy 2 → no silencing or severely reduced silencing efficacy	1 → slightly reduced silencing efficacy 2 → significantly reduced silencing efficacy	1 → no effect or slightly reduced silencing efficacy	Effect of two mutations was most pronounced when one was in the centre and the other in the 3� end.	342
Insect� embryo extract	Synthetic siRNA, target cleavage monitored	combination of 7-9 3� mismatches with 1-2 5� mismatches → no target cleavage	up to 9 → cleavage of target increasingly slowed down 10 → no target cleavage		up to 5 → cleavage of target increasingly slowed down 6 → no target cleavage	Target cleavage with 9 3� mismatches was slightly enhanced by combining with one 5� mismatch.	145
Isolated mammalian cells	Synthetic siRNAs, analysed messenger RNA expression patterns (microarray assay)		1 → reduced silencing efficacy		1 → reduced silencing efficacy		69

* Some studies report the overall effect of mismatches regardless of their positions - these results are summarised under �all regions� in the table. The 3� and 5� ends are those of the siRNA. The 3� end of the siRNA is the 5� end of the target site on the messenger RNA and vice versa. Effects of mismatches in more than one region are independent of each other unless otherwise stated.

B) Experiments involving large scale expression profiling

Organism	Experiment	Requirements for the siRNA-target interaction to induce silencing*				Comments	References
		all regions	3� end	centre	5�end		
Isolated mammalian cells	Synthetic siRNAs, analysis messenger RNA expression patterns (microarray assay)	15 matches in total with at least 11 contiguous matches.		14-15 matches, encompassing the centre. Having one mismatch in the 3� half abolished silencing for this subgroup.	At least 9 matches (including the centre) and 1 additional match in the 3� end.	Some messenger RNAs with short stretches of <8 nucleobases similarity to the siRNAs were affected but these were most likely secondary effects not triggered by a direct interaction with the siRNAs.	69
Plant	Overexpression of four natural plant miRNAs, analysis of messenger RNA expression patterns (microarray assay)	No more than two contiguous internal mismatches.	No more than three mismatches even if there is a stretch of 10 or more consecutive matches in the 5� region.	No mismatches at positions 10 or 11.	Not more than one mismatch in positions 2-12 from the miRNA 5� end.	A few exceptions to the rules inferred from overexpression of the four chosen miRNAs have been reported [162,343,344]. Conversely, some messenger RNAs that had valid target sites according to the rules found in this study were not affected by overexpression of the matching miRNAs.	156

* In contrast to table 2A, the results in table 2B were obtained from large scale expression profiling analyses. Rather than examining the effect of mismatches on target messenger RNA silencing, these data give an indication of the number and positions of matches that are sufficient to induce silencing of a messenger RNA.

5� and 3� ends

The two ends of a DNA or RNA molecule are not equal. Nucleotides, the building blocks of DNA or RNA, are linked by a ribose backbone. Ribose is a carbohydrate that contains five carbon atoms that form a ring structure. In biochemistry these are referred to as carbon atoms 1� to 5�. To link two units of ribose, the hydroxyl (OH) group of a 3� carbon atom reacts with the phosphate group of a 5� carbon atom.
In the final DNA polymer (chain), the terminal ribose at one end has a free hydroxyl group at a 3� carbon atom, while the other end carries a free 5� phosphate group. The two ends are referred to as the 3� and 5� end respectively. As a result, DNA or RNA molecules have a polarity which is recognised by enzymes that interact with them. For example: the sequence 5�-ACTG-3� is *not* identical to 3�-ACTG-5� and a protein that binds to DNA with the former sequence will not accept the latter. Sequences are always written down from the 5� to the 3� end.
The two ends of messenger RNA molecules are usually modified with structures, termed �5�cap� and �3� poly(A) tail�, that play a role in the translation process. A lack of these structures can make the �aberrant� RNA a target of the RNA silencing machinery by attracting an RNA-polymerase that converts the single-stranded messenger RNA into a double-stranded substrate for Dicer [1] .

Literature

1.�������� Gazzani, S., Lawrenson, T., Woodward, C., Headon, D. & Sablowski, R. (2004). A link between mRNA turnover and RNA interference in Arabidopsis. *Science* **306**: 1046-1048

Wobble basepairs

Although, in RNA, uracil (U) normally forms basepairs with adenine (A), so-called �wobble� basepairs of uracil (U) with guanine (G) can also occur. Unlike complete mismatches, wobble basepairs do not disturb the spatial geometry of the double helix. Wobble mismatches between siRNAs and target sites can be well tolerated in some cases [1] .

Literature

1.��������� Du, Q., Thonberg, H., Wang, J., Wahlestedt, C. & Liang, Z. (2005). A systematic analysis of the silencing effects of an active siRNA at all single-nucleotide mismatched target sites. *Nucleic Acids Res* **33**: 1671-1677

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Domain

Proteins are made up of amino acid polymers that from three-dimensional structures, which determine the enzymatic or structural function of the protein. While some of these structures are peculiar to a single protein, others can be found in many different proteins. These modules of distinct self-stabilizing structures are referred to as �domains�.� DNA or RNA binding domains, for example, can be found in many proteins that need to interact with nucleic acids.

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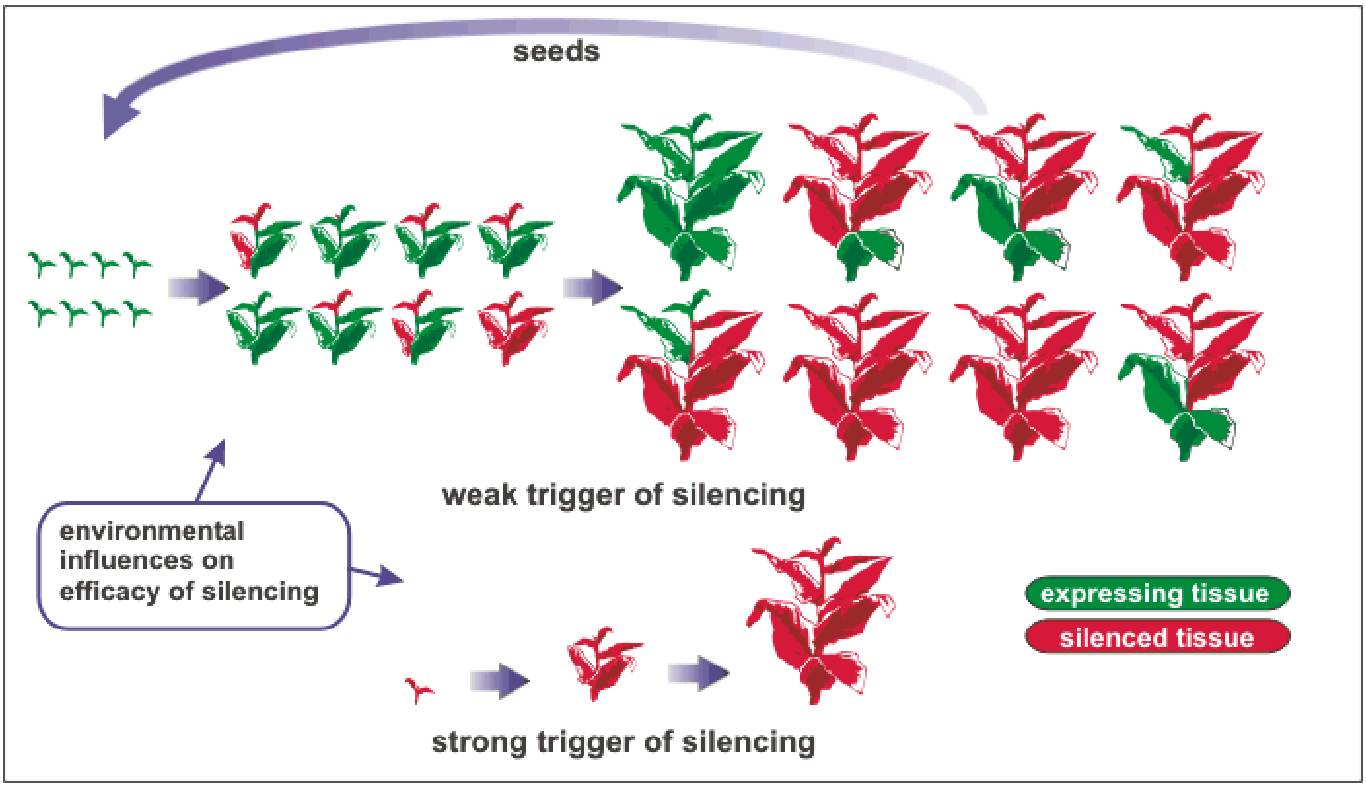
Hypothetical hazard: variability of onset and extend of RNA silencing



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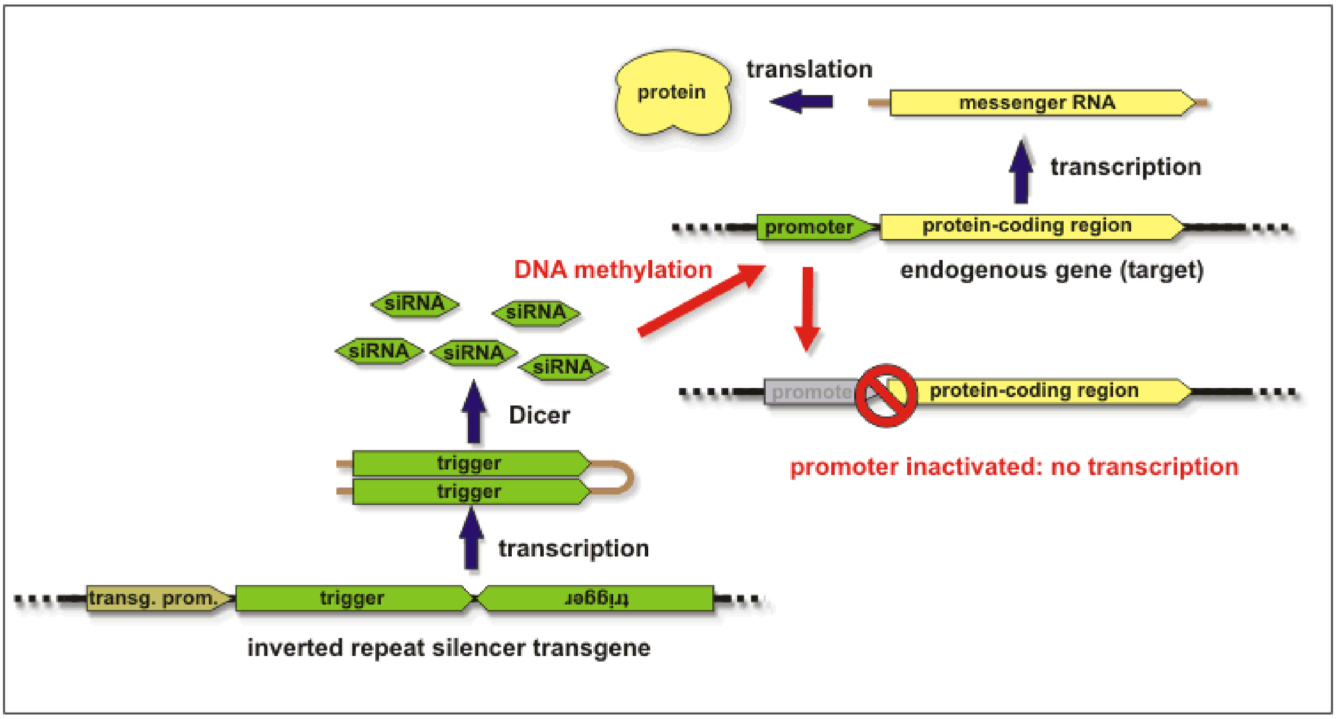
Silencing a gene with additional copies in sense or antisense orientation (co- or antisense-suppression) can lead to unreliable triggering of silencing in late stages of the plant's development. With these "weak" triggers of silencing, silenced and non-silenced tissue is frequently found on one plant and some plants often fail to initiate silencing altogether.

Strong triggers such as double-stranded RNA normally induce silencing in the seedling and maintain the silenced state throughout the plant's life time.

Environmental parameters can have an influence on the efficacy of RNA silencing.

Post-transcriptional silencing is not inheritable. The silenced state is lost during reproduction and re-established with the same frequency and spatial/temporal pattern in the next generation.

Modes of transgene-induced silencing: post-transcriptional / transcriptional (2 slides)



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Transgene-induced transcriptional silencing

As in slide 1, silencing is triggered by an inverted repeat construct. In this case, however, the sequence of the inverted repeat matches the promoter of the target gene and not its protein-coding region. The siRNAs generated from this trigger induce RNA-directed DNA methylation of the promoter sequence. This process inactivates the promoter, thus abolishing transcription of the messenger RNA.

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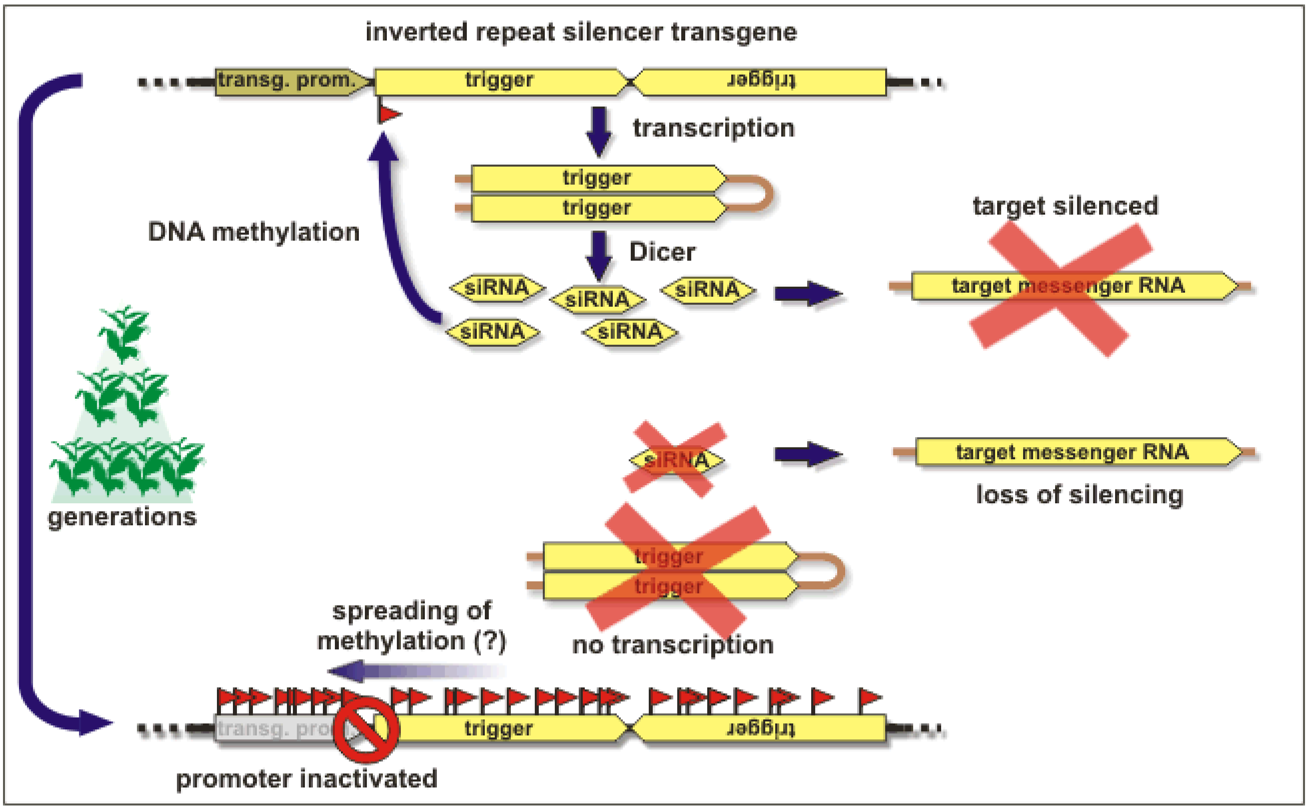
Hypothetical hazard: instability of silencing over generations



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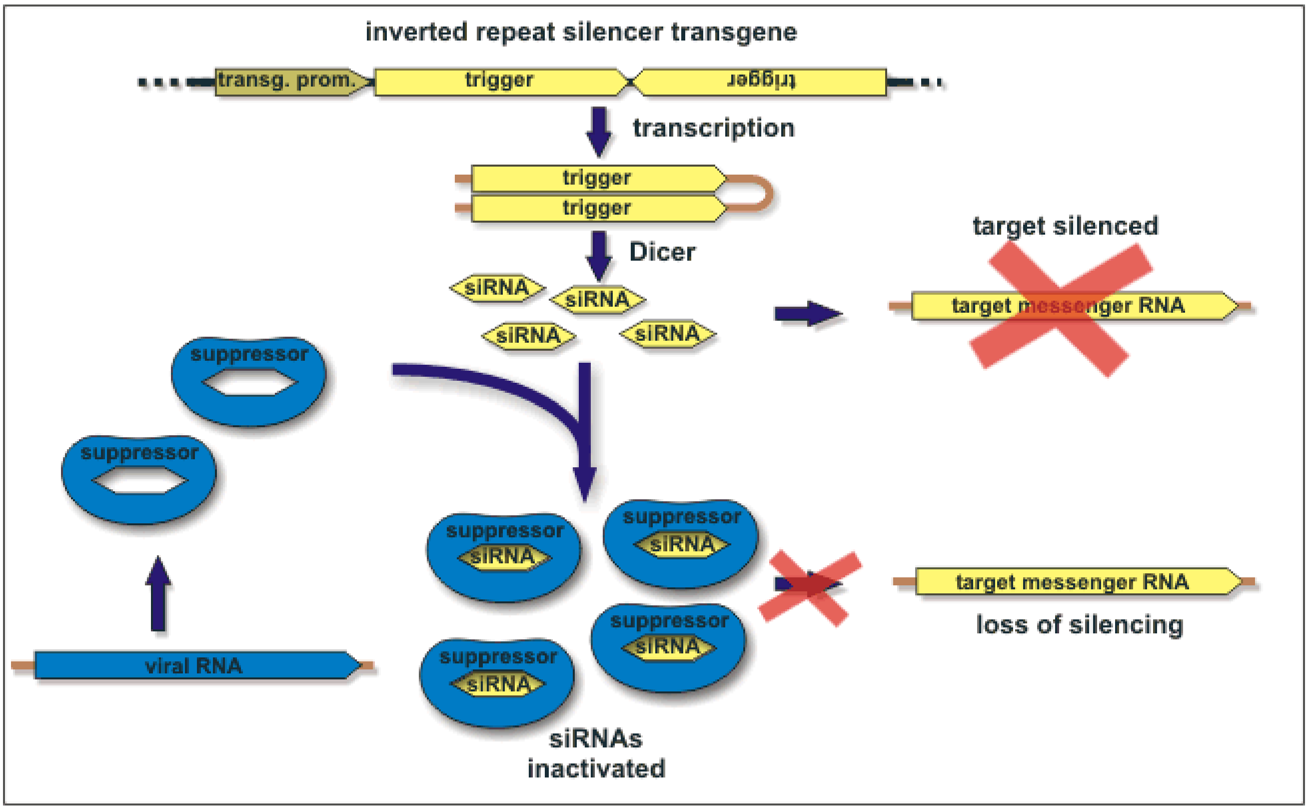
A silencing trigger, double-stranded RNA in the example shown here, directs siRNA-mediated degradation of target messenger RNA and methylation of its own copy in the plant's genome. The methylation (shown here as red flags) can accumulate and spread throughout the transcribed region but does not easily spread into the promoter (labelled "transg. prom.") that controls the expression of the transgene. If methylation does spread into the promoter, the trigger is no longer transcribed, resulting in a loss of siRNAs and re-activation of the silenced target gene. This process might take several generations to manifest.

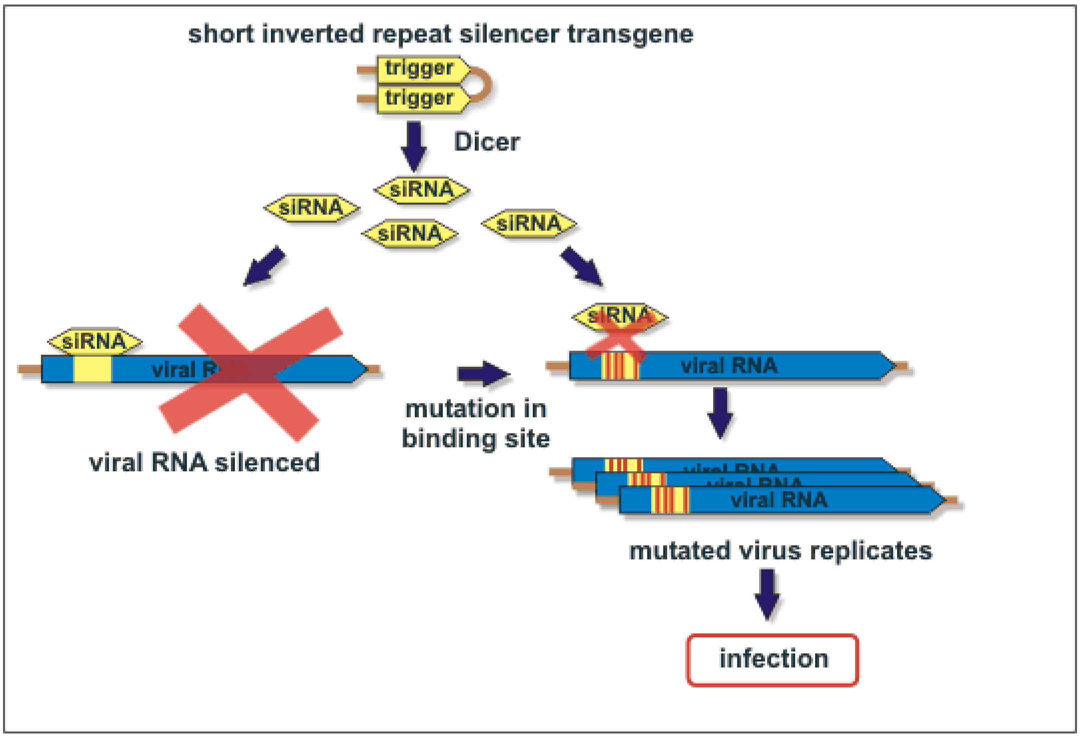
Hypothetical hazard: suppression of RNA silencing by viruses



Many plant viruses inhibit RNA silencing by encoding their own silencing suppressor proteins. Silencing suppressors have evolved independently in various taxonomical groups of viruses. Consequently, they have different modes of action and interfere with RNA silencing pathways at different steps. The example here shows a suppressor that binds siRNAs to inactivate them. This is the mode of action of the tombusvirus P19 protein, one of the most extensively studied viral suppressors.

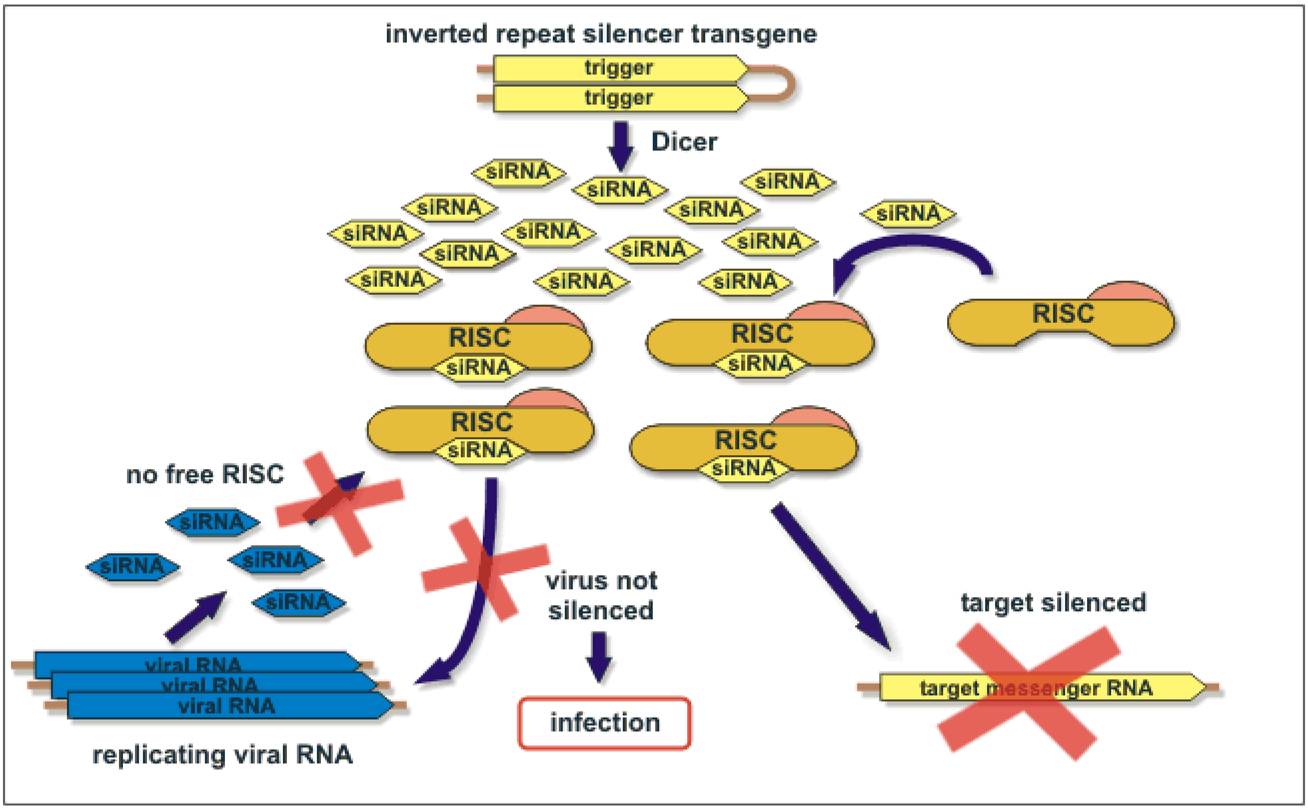
A viral infection could result in a loss of silencing of the target gene by the action of silencing suppressor proteins.

Hypothetical hazard: escape of viruses from silencing-based resistance



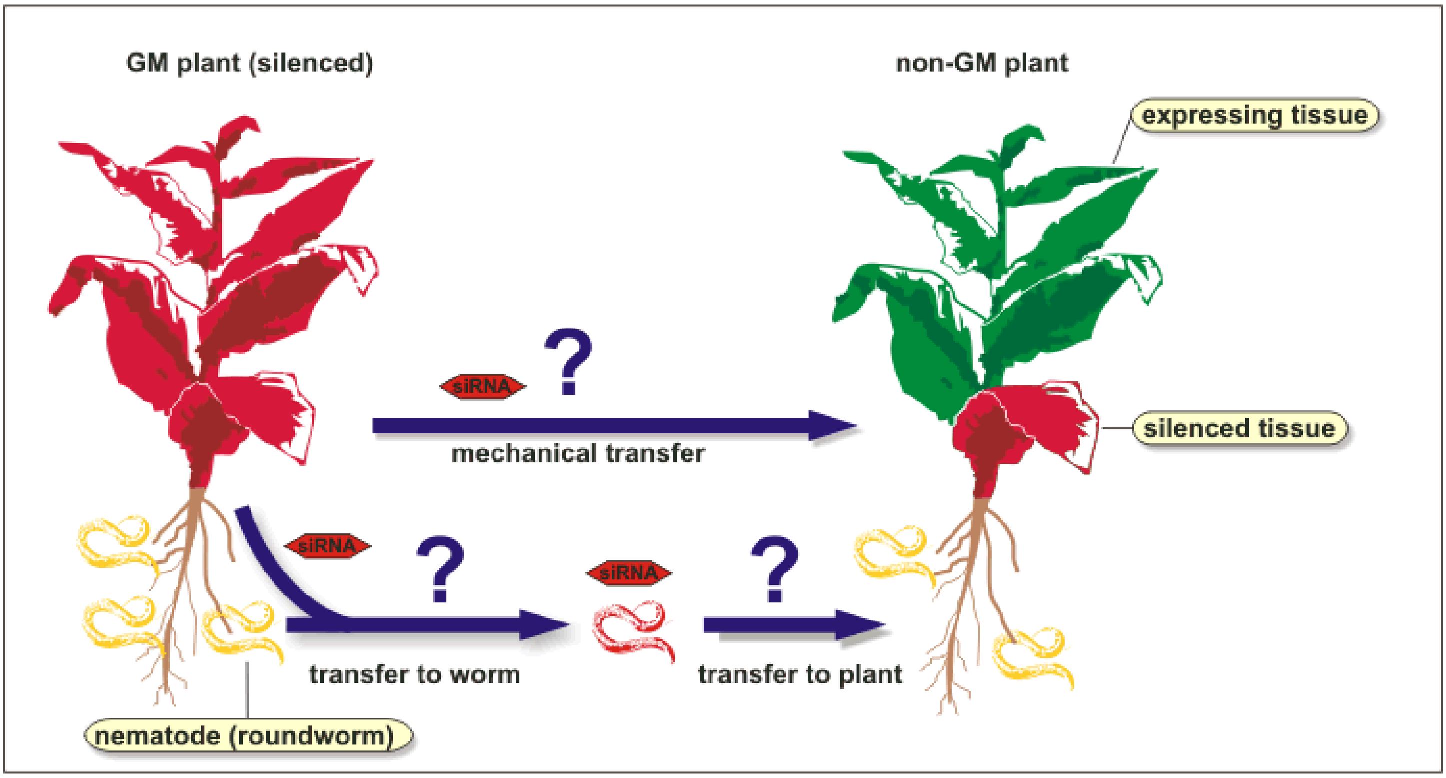
Viruses that are targeted for RNA silencing by short triggers such as short hairpin (sh)RNAs can escape the silencing-based resistance by acquiring mutations within the target region, thus impairing the interaction between the siRNA(shRNA) and the viral RNA. The longer the trigger, the more difficult it is for the virus to acquire the necessary number of mutations without affecting its viability.

Hypothetical hazards: saturation of the silencing machinery



Overexpressed potent triggers of RNA silencing could saturate the silencing machinery by loading all available units of the effector complex RISC with transgene-derived siRNAs.
A virus that is normally fought by RNA silencing can accumulate to high levels in a plant with a saturated silencing machinery, thus causing severe infections.

Hypothetical hazard: horizontal transfer of RNA silencing



RNA silencing is a highly conserved mechanism in plants, animals and fungi. If triggers or mediators of RNA silencing, such as siRNAs, could be transferred from a GM plant to other organisms in a functional state, they could induce silencing of any sufficiently matching genes in the receiving organism.

Some plant viruses are transferred from plant to plant by mechanical inoculation. Thus, there could be a mechanical transfer of silencing triggers or siRNAs between plants, although this is a very unlikely scenario.

The nematode species *C. elegans* is an important model organism for the study of RNA silencing. Silencing can be induced in these worms by feeding them on bacteria that produce the silencing trigger. Since many nematodes live in the rhizosphere it is conceivable that these could pick up RNA triggers or siRNAs from the plant. This could result in silencing in the worm if there are sufficiently matching nematode genes. Vice versa, a "silenced" worm might induce silencing in a non-GM plant by feeding on its roots. So far, there is no experimental indication that this unlikely event is possible in nature but silencing can be triggered in a plant by rubbing RNA extracts from silenced plants onto non-silenced leaves.

Position effect variegation/ position effect silencing

Following chromosomal rearrangement events, euchromatic genes can end up in juxtaposition to heterochromatic regions. The heterochromatic state can then spread into the formerly euchromatic gene, thus silencing it. This is referred to as �position effect silencing�. The resulting phenotype often is a mosaic pattern as this type of silencing is variable between individual cells. Later in development, the expression status of the gene becomes clonally stable, giving rise to patches of similarly expressing cells. This is known as �position effect variegation� (PEV).
Interestingly, some *Drosophila* genes that normally reside in heterochromatic regions exhibit PEV when moved far away from heterochromatin, suggesting that genes are optimised for expression in their �home environment� and that heterochromatic regions are not always inactive [1,2]

Literature

1.�������� Wakimoto, B. T. & Hearn, M. G. (1990). The effects of chromosome rearrangements on the expression of heterochromatic genes in chromosome 2L of Drosophila melanogaster. *Genetics* **125**: 141-154

2.�������� Eberl, D. F., Duyf, B. J. & Hilliker, A. J. (1993). The role of heterochromatin in the expression of a heterochromatic gene, the rolled locus of Drosophila melanogaster. *Genetics* **134**: 277-292

AGO1 (ARGONAUTE1), *Arabidopsis thaliana* protein

The Arabidopsis AGO1 protein was the first identified member of the extensive and highly conserved ARGONAUTE family and it is now known to play a crucial role in the RNA silencing machinery. AGO1 contributes a nuclease activity to the silencing effector complex RISC, which carries out the siRNA-guided cleavage of the target RNA [1,2] .
Several other members of the ARGONAUTE protein family also have the structure that is required for a nuclease activity [1] and AGO1 does not associate with all siRNA that are produced in a cell. Therefore it is expected, that other ARGONAUTE proteins perform the cleavage reaction in different silencing pathways.
Recent findings show that AGO1 binds siRNAs, performs the cleavage reaction and does not appear to be part of a complex when purified from plant extracts. Therefore it is conceivable that there is no RISC complex as such in plants and that AGO1 *is* RISC [1] .�

Literature

1.�������� Baumberger, N. & Baulcombe, D. C. (2005). Arabidopsis ARGONAUTE1 is an RNA Slicer that selectively recruits microRNAs and short interfering RNAs. *Proc Natl Acad Sci USA* **102**: 11928-11933

2.�������� Qi, Y., Denli, A. M. & Hannon, G. J. (2005). Biochemical specialization within Arabidopsis RNA silencing pathways. *Mol Cell* **19**: 421-428

AGO4 (ARGONAUTE4), *Arabidopsis thaliana* protein

AGO4, a member of the ARGONAUTE protein family in *Arabidopsis thaliana*, has been implicated in transcriptional silencing pathways and RNA-directed DNA-methylation [1,2] . In double-stranded-RNA induced silencing, AGO4 is required for the maintenance but not the initiation of DNA methylation [2] .

Literature

1.�������� Zilberman, D., Cao, X. & Jacobsen, S. E. (2003). *ARGONAUTE4* control of locus specific siRNA accumulation and DNA and histone methylation. *Science* **299**: 716-719

2.�������� Zilberman, D., Cao, X., Johansen, L. K., Xie, Z., Carrington, J. C. & Jacobsen, S. E. (2004). Role of *Arabidopsis ARGONAUTE4* in RNA-Directed DNA Methylation Triggered by Inverted Repeats. *Curr Biol* **14**: 1214-1220

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Chromodomain

The chromatin organization modifier (chromo) domain binds to methylated histones. Chromodomain proteins catalyse the transition from euchromatin to heterochromatin.

DCL1, Dicer-like1 (*Arabidopsis thaliana*)

The *Arabidopsis* enzyme DCL1 is one of the four Dicer-like proteins in this plant species. It is required for miRNA accumulation (Figure), where it is involved in at least two steps of the miRNA maturation pathway [1,2] . However, DCL1 is not involved in siRNA production from double-stranded triggers of post-transcriptional gene silencing [3] . The accumulation of DCL1 is itself regulated by a miRNA in a feed-back loop: an increase in DCL1 abundance leads to a higher production rate of miRNAs, which in turn reduce the rate of DCL1 production [4] . In contrast to its animal homologues, DCL1 is located in the nucleus of the cell, suggesting that miRNA maturation in plants occurs in the nucleus [5,6] .
Recent biochemical analyses have confirmed that DCL1 processes double-stranded RNA into 21 nucleotide long siRNAs, whereas DCL3 is the major Dicer activity producing the longer (24-25 nucleotide) siRNAs [7] . These two Dicer-like enzymes reside in different complexes of unknown composition [7] . HYL1, a double-stranded RNA binding protein, is so far the only identified component of the DCL1 complex [8] .

Literature

1.��������� Park, W., Li, J., Song, R., Messing, J. & Chen, X. (2002). CARPEL FACTORY. a Dicer homolog, and HEN1, a novel protein, act in microRNA metabolism in *Arabidopsis thaliana*. *Curr Biol* **12**: 1484-1495

2.��������� Kurihara, Y. & Watanabe, Y. (2004). Arabidopsis micro-RNA biogenesis through Dicer-like 1 protein functions. *Proc Natl Acad Sci USA* **101**: 12753-12758

3.��������� Finnegan, E. J., Margis, R. & Waterhouse, P. M. (2003). Posttranscriptional gene silencing is not compromised in the *Arabidopsis CARPEL FACTORY* (*DICER-LIKE1*) mutant, a homolog of Dicer-1 from *Drosophila*. *Curr Biol* **13**: 236-240

4.��������� Xie, Z., Kasschau, K. D. & Carrington, J. C. (2003). Negative Feedback Regulation of *Dicer-Like1* in *Arabidopsis* by microRNA-Guided mRNA Degradation. *Curr Biol* **13**: 784-789

5.��������� Xie, Z., Johansen, L. K., Gustafson, A. M., Kasschau, K. D., Lellis, A. D., Zilberman, D., Jacobsen, S. E. *et al.* (2004). Genetic and Functional Diversification of Small RNA Pathways in Plants. *PLoS Biology* **2**: 642-652

6.��������� Papp, I., Mette, F., Aufsatz, W., Daxinger, L., Schauer, S. E., Ray, A., van der Winden, J. *et al.* (2003). Evidence for Nuclear Processing of Plant Micro RNA and Short Interfering RNA Precursors. *Plant Physiol* **132**: 1382-1390

7.��������� Qi, Y., Denli, A. M. & Hannon, G. J. (2005). Biochemical specialization within Arabidopsis RNA silencing pathways. *Mol Cell* **19**: 421-428

8.��������� Hiraguri, A., Itoh, R., Kondo, N., Nomura, Y., Aizawa, D., Murai, Y., Koiwa, H. *et al.* (2005). Specific interactions between Dicer-like proteins and HYL1/DRB-family dsRNA-binding proteins in Arabidopsis thaliana. *Plant Mol Biol* **57**: 173-188

DCL2, Dicer-like2 (*Arabidopsis thaliana*)

According to a recent biochemical study, DCL2 does not contribute significantly to siRNA production when a non-viral double-stranded RNA is used as a trigger for RNA silencing [1] . In contrast, DCL2 has been reported to be associated with siRNA production from at least some plant viruses [2] .

Literature

1.�������� Qi, Y., Denli, A. M. & Hannon, G. J. (2005). Biochemical specialization within Arabidopsis RNA silencing pathways. *Mol Cell* **19**: 421-428

2.�������� Xie, Z., Johansen, L. K., Gustafson, A. M., Kasschau, K. D., Lellis, A. D., Zilberman, D., Jacobsen, S. E. *et al.* (2004). Genetic and Functional Diversification of Small RNA Pathways in Plants. *PLoS Biology* **2**: 642-652

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DCL3, Dicer-like3 (*Arabidopsis*)

DCL3, one of the four Dicer enzymes in *Arabidopsis*, is required for the biosynthesis of endogenous siRNAs [1] . A recent biochemical analysis in *Arabidopsis* has shown that DCL3 is responsible for producing the longer size-class (24-25 nucleotides) of siRNAs [2] , which are known to be required for systemic silencing and RNA-directed DNA methylation [3,4] .

Literature

1.�������� Xie, Z., Johansen, L. K., Gustafson, A. M., Kasschau, K. D., Lellis, A. D., Zilberman, D., Jacobsen, S. E. *et al.* (2004). Genetic and Functional Diversification of Small RNA Pathways in Plants. *PLoS Biology* **2**: 642-652

2.�������� Qi, Y., Denli, A. M. & Hannon, G. J. (2005). Biochemical specialization within Arabidopsis RNA silencing pathways. *Mol Cell* **19**: 421-428

3.�������� Hamilton, A. J., Voinnet, O., Chappell, L. & Baulcombe, D. C. (2002). Two classes of short interfering RNA in RNA silencing. *EMBO J* **21**: 4671-4679

4.�������� Baulcombe, D. (2004). RNA silencing in plants. *Nature* **431**: 356-363

DCL4, Dicer-like4 (*Arabidopsis thaliana*)

In addition to miRNAs, plants have been shown to encode other regulatory small RNAs, called trans-acting (ta)-siRNAs. The *Arabidopsis* enzyme DCL4, a member of the Dicer family, processes double-stranded precursor RNAs into ta-siRNAs [1,2] . DCL4 forms a complex which includes at least one more double-stranded RNA binding protein [3] .

In a recent study, DCL4 was also identified as the Dicer activity that produces siRNAs from long double-stranded transgene RNA, commonly used to trigger silencing in transgenic plants. Furthermore, this study demonstrated that 21 nucleotide long siRNAs produced by DCL4 are the signal that enables cell-to-cell movement of RNA silencing in plants [4] .

Literature

1.��������� Xie, Z., Allen, E., Wilken, A. & Carrington, J. C. (2005). DICER-LIKE 4 functions in trans-acting small interfering RNA biogenesis and vegetative phase change in Arabidopsis thaliana. *Proc Natl Acad Sci USA* **102**: 12984-12989

2.��������� Yoshikawa, M., Peragine, A., Park, M. Y. & Poethig, R. S. (2005). A pathway for the biogenesis of trans-acting siRNAs in Arabidopsis. *Genes Dev* **19**: 2164-2175

3.��������� Hiraguri, A., Itoh, R., Kondo, N., Nomura, Y., Aizawa, D., Murai, Y., Koiwa, H. *et al.* (2005). Specific interactions between Dicer-like proteins and HYL1/DRB-family dsRNA-binding proteins in Arabidopsis thaliana. *Plant Mol Biol* **57**: 173-188

4.��������� Dunoyer, P., Himber, C. & Voinnet, O. (2005). DICER-LIKE 4 is required for RNA interference and produces the 21-nucleotide small interfering RNA component of the plant cell-to-cell silencing signal. *Nature Genetics* **Published online ahead of print 6 November 2005**

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DDM1 (*Arabidopsis thaliana*)

The *Arabidopsis* protein DDM1 is a chromatin remodelling enzyme that is required for both DNA and histone methylation. [1,2] .

Literature

1.��������� Jeddeloh, J. A., Bender, J. & Richards, E. J. (1998). The DNA methylation locus DDM1 is required for maintenance of gene silencing in Arabidopsis. *Genes Dev* **12**: 1714-1725

2.��������� Gendrel, A. V., Lippman, Z., Yordan, C., Colot, V. & Martienssen, R. (2002). Dependence of Heterochromatic Histone H3 Methylation Patterns on the Arabidopsis Gene DDM1. *Science* **297**: 1871-1873

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Drosophila melanogaster

The fruitfly *Drosophila melanogaster* is an important and well-studied model organism for genetics and molecular biology.

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egs1 and egs2

Mutations in these two genes enhance RNA silencing in *Arabidopsis*. The proteins encoded by these two genes are therefore expected to be negative regulators of RNA silencing but, despite being the earliest RNA silencing mutations described in plants, their exact nature has not been resolved yet [1]. Another negative regulator of RNA silencing, rgs-CaM, has been identified in tobacco [2]

Literature

1.��������� Dehio, C. & Schell, J. (1994). Identification of plant genetic loci involved in posttranscriptional mechanism for meiotically reversible transgene silencing. *Proc Natl Acad Sci USA* **91**: 5538-5542

2.��������� Anandalakshmi, R., Marathe, R., Ge, X., Herr Jr., J. M., Mau, C., Mallory, A., Pruss, G. *et al.* (2000). A Calmodulin-Related Protein That Suppresses Posttranscriptional Gene Silencing in Plants. *Science* **290**: 142-144

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Heterochromatinisation

The process of increasing the �packing density� of chromatin, which reduces the transcriptional activity of the affected region of DNA, is called heterochromatinisation.

Homology

In molecular biology, sequences of nucleobases in DNA/RNA and sequences of amino acids in proteins are often compared to one another to fathom their relationship and functional similarity. Two sequences that have common ancestry or are functionally similar are �homologous� to each other. This does not imply that the sequences themselves are identical and, in fact, the proportion of identical nucleobases or amino acids in homologous genes/proteins can be very low.

DNA sequences encode the amino acid sequences of proteins in �triplets�, i.e. three consecutive nucleobases encode one aminoacid. The genetic code is somewhat redundant, because most aminoacids can be encoded by more than one triplet. As a result, some mutations change the DNA sequence without altering the aminoacid sequence of the protein product because the changed triplet still encodes the same aminoacid. In addition, different amino acids are often functionally similar to each other so that a mutation can change an amino acid without changing the structure and thus the function of the domain it resides in. Molecular biologists therefore examine the similarity of proteins, or domains of proteins, to find out whether or not they are homologues of each other. If they are, the genes encoding them are homologues although their actual nucleobase sequences can be very different from each other.

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HYL1

The *Arabidopsis* HYL1 (HYPONASTIC LEAVES1) protein is involved in miRNA production but not in post-transcriptional transgene silencing [1,2] . Neither HYL1 nor HEN1 are absolutely required for miRNA biosynthesis but if both are missing the plant is infertile, suggesting a synergistic effect of these two proteins [1] . HYL1 is homologous to the *Drosophila melanogaster* protein R2D2, which probes the ends of the double-stranded small RNA. This process determines which of the two strands of the small RNA is to be incorporated into the silencing effector complex RISC [1,2,3] . The non-incorporated strand is discarded and degraded.
Like R2D2, HYL1 has a double-stranded RNA binding domain and it has been shown recently to form complexes with the Dicer enzyme DCL1 [4] . However, some important differences make it seem unlikely that HYL1 has exactly the same role in plants as R2D2 in animals [2] .

Literature

1.�������� Vazquez, F., Gasciolli, V., Crete, P. & Vaucheret, H. (2004). The Nuclear dsRNA Binding Protein HYL1 is Required for MicroRNA Accumulation and Plant Development, but not Posttranscriptional Transgene Silencing. *Curr Biol* **14**: 346-351

2.�������� Han, M.-H., Goud, S., Song, L. & Fedoroff, N. (2004). The *Arabidopsis* double-stranded RNA-binding protein HYL1 plays a role in microRNA-mediated gene regulation. *Proc Natl Acad Sci USA* **101**: 1093-1098

3.�������� Lu, C. & Fedoroff, N. (2000). A mutation in the Arabidopsis HYL1 gene encoding a dsRNA binding protein affects responses to abscisic acid, auxin, and cytokinin. *Plant Cell* **12**: 2351-2366

4.�������� Hiraguri, A., Itoh, R., Kondo, N., Nomura, Y., Aizawa, D., Murai, Y., Koiwa, H. *et al.* (2005). Specific interactions between Dicer-like proteins and HYL1/DRB-family dsRNA-binding proteins in Arabidopsis thaliana. *Plant Mol Biol* **57**: 173-188

Genomic imprinting

Sexually reproducing organisms inherit two copies of each gene � one from each of the two parents. In some cases, only one of the two copies is active, while the other one is inactivated by DNA methylation. This phenomenon was first reported in insects in the late 1950s but is now known to occur in mammals and flowering plants as well [1,2,3] . Having two copies of a gene should protect the organism from detrimental mutations in one of the copies, because the mutation can be compensated by the second, intact, copy of the gene. From this point of view it seems counter-productive to inactivate the �backup� copy by genomic imprinting.

The most widely accepted theory to explain genomic imprinting implies a �battle of the sexes�: females, especially female mammals, invest a lot into the pre-natal development of the embryo and it is beneficial for them to strike a balance between this investment and their own physical fitness to ensure multiple births and thus more offspring. The male parent, in contrast, would profit from forcing the female mate to invest more into the embryo to give his offspring a better chance of survival. Thus, the maternally inherited half of the genome reduces embryo growth while the paternal half promotes it. As a result of this �parental gene conflict�, many genes that are implicated in the growth and development of the mammalian foetus or placenta are subject to imprinting.

In mice, approximately 80 imprinted genes have been identified so far and a similar number is expected in humans (http://www.mgu.har.mrc.ac.uk/research/imprinting/).

Literature

1.�������� McDonald, J. F., Matzke, M. A. & Matzke, A. J. (2005). Host defenses to transposable elements and the evolution of genomic imprinting. *Cytogenet Genome Res* **110**: 242-249

2.�������� Kohler, C. & Grossniklaus, U. (2005). Seed development and genomic imprinting in plants. *Prog Mol Subcell Biol* **38**: 237-262

3.�������� Autran, D., Huanca-Mamani, W. & Vielle-Calzada, J. P. (2005). Genomic imprinting in plants: the epigenetic version of an Oedipus complex. *Curr Opin Plant Biol* **8**: 19-25

Methyltransferases

A methyltransferase is an enzyme that adds methyl groups to its substrate (see methylation). Cytosine methyltransferases methylate cytosine residues in DNA molecules. In plants, there are three distinct classes of cytosine methyltransferases known as MET, CMT and DRM. MET1, a member of the MET class, is the major enzyme responsible for the maintenance of CG methylation in plants [1] . CMT3, a CMT class methyltransferase, is a major enzyme for the non-CG methylation maintenance. CMT3 and DRM methyltransferases are responsible for establishing new DNA-methylation patterns [2] .

Literature

1.�������� Jones, L., Ratcliff, F. & Baulcombe, D. C. (2001). RNA-directed transcriptional gene silencing in plants can be inherited independently of the RNA trigger and requires Met1 for maintenance. *Curr Biol* **11**: 747-757

2.�������� Bender, J. (2004). DNA Methylation and Epigenetics. *Annu Rev Plant� Biol* **55**: 41-68

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Nuclease, RNase, DNase

An enzyme that degrades nucleic acids is called a nuclease. An RNase degrades RNA and a DNase degrades DNA.

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Primer

DNA or RNA polymerases synthesise DNA/RNA that is complementary in sequence to a pre-existing template strand of DNA/RNA. A primer is a short fragment of DNA or RNA that binds to the template strand. The polymerase then extends the 3� end of the primer until it reaches the end of the template strand or is stopped by other means. Many, but not all, polymerases require such a primer and some can perform both primed and unprimed reactions [1] .

Literature

1.�������� Makeyev, E. V. & Bamford, D. H. (2002). Cellular RNA-dependent RNA polymerase involved in posttranscriptional gene silencing has two distinct activity modes. *Mol Cell* **10**: 1417-1427

R2D2

In the fruitfly *Drosophila melanogaster*, this protein probes the double-stranded siRNA to find the �stronger� end, thus determining which of the two strands is incorporated into RISC to guide target cleavage [1] .

Literature

1.�������� Tomari, Y., Matrange, C., Haley, B., Martinez, N. & Zamore, P. D. (2004). A Protein Sensor for siRNA Asymmetry. *Science* **306**: 1377-1380

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RNA-dependent RNA-polymerase 1 (RDR1), *Arabidopsis thaliana*

RDR1 is one of six RNA-dependent RNA-polymerases encoded by the *A. thaliana* genome. RDR1 has been shown to participate in silencing pathways that target viruses in several plant species [1,2,3] . The production of RDR1 is induced by salicylic acid, a known signal molecule involved in plant defence pathways [4,5] .

Literature

1.��������� Xie, Z., Fan, B., Chen, C. H. & Chen, Z. (2001). An important role of an inducible RNA-dependent RNA polymerase in plant antiviral defense. *Proc Natl Acad Sci USA* **98**: 6516-6521

2.��������� Yu, D., Fan, B., MacFarlane, S. A. & Chen, Z. (2003). Analysis of the involvement of an inducible *Arabidopsis* RNA-dependent RNA polymerase in antiviral defense. *Mol Plant Microbe Interact* **16**: 206-216

3.��������� Yang, S. J., Carter, S. A., Cole, A. B., Cheng, N. H. & Nelson, R. S. (2004). A natural variant of a host RNA-dependent RNA polymerase is associated with increased susceptibility to viruses by Nicotiana benthamiana. *Proc Natl Acad Sci USA* **101**: 6297-6302

4.��������� Chivasa, S., Murphy, A. M., Naylor, M. & Carr, J. P. (1997). Salicylic Acid Interferes with Tobacco Mosaic Virus Replication via a Novel Salicylhydroxamic Acid-Sensitive Mechanism. *Plant Cell* **9**: 547-557

5.��������� Naylor, M., Murphy, A. M., Berry, J. O. & Carr, J. P. (1998). Salicylic Acid Can Induce Resistance to Plant Virus Movement. *Mol Plant Microbe Interact* **11**: 860-868

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RNA-dependent RNA-polymerase 2 (RDR2), *Arabidopsis thaliana*

RDR2 is one of six RNA-dependent RNA-polymerases encoded by the *A. thaliana* genome. It cooperates with the DNA-dependent RNA-polymerase 4� to produce endogenous siRNAs [1,2]. Some genomic regions are believed to attract DNA-dependent RNA-polymerase 4 which produces transcripts that are subsequently converted to the double-stranded form by RDR2 [2]. However, RDR2 seems to be required even in cases were the original transcript could form a double-stranded structure on its own [1].
The same pathway producing endogenous siRNAs may also be responsible for the trigger-independent maintenance of transgene silencing by attracting DNA-dependent RNA-polymerase 4 to the methylated integration site of the transgene in the plant genome. The transcripts provided by this enzyme are processed into siRNAs by RDR2 and a Dicer enzyme. These siRNAs direct the methylation of the transgene which enforces transcription by DNA-dependent RNA-polymerase 4. The result is a self-sustaining feed-back loop that maintains the silenced state of the transgene [3].

Literature

1.�������� Xie, Z., Johansen, L. K., Gustafson, A. M., Kasschau, K. D., Lellis, A. D., Zilberman, D., Jacobsen, S. E. *et al.* (2004). Genetic and Functional Diversification of Small RNA Pathways in Plants. *PLoS Biology* **2**: 642-652

2.�������� Herr, A. J., Jensen, M. B., Dalmay, T. & Baulcombe, D. (2005). RNA polymerase IV directs silencing of endogenous DNA. *Science* **308**: 118-120

3.�������� Herr, A. J. (2005). Pathways through the small RNA world of plants. *FEBS Letters* **579**: 5879-5888

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RNA-dependent RNA-polymerase 6 (RDR6), *Arabidopsis thaliana*

RDR6, also known as SDE1 and SGS2, is one of six RNA-dependent RNA-polymerases encoded by the *A. thaliana* genome. This enzyme was one of the first to be identified as a component of the RNA silencing machinery in plants by screening mutant plants deficient in RNA silencing [1,2] . Without RDR6, RNA silencing can still be triggered by double-stranded but not single-stranded RNA, showing that this enzyme is responsible for converting single-stranded RNA into the double-stranded form by synthesising a complementary strand. It is not clear how RDR6 recognises single-stranded RNA but missing end structures have been shown to be one possibility to mark out an RNA as �aberrant� and thus as a target of RNA silencing [3] .

RDR6 is also required for the reception, but not the production, of the long-range RNA systemic silencing signal (see Figure), while having no effect on the short-range signal [4,5] . It has been suggested that RDR6 is involved in antiviral defence because it enables systemic signalling that can inhibit viral spread by targeting the virus in the early stages of its infection cycle [5] .

Recently, RDR6 has also been implied in the biosynthesis of trans-acting endogenous siRNAs, which have a role in developmental regulation [6] .

Literature

1.��������� Dalmay, T., Hamilton, A. J., Rudd, S., Angell, S. & Baulcombe, D. C. (2000). An RNA-dependent RNA polymerase gene in *Arabidopsis* is required for posttranscriptional gene silencing mediated by a transgene but not by a virus. *Cell* **101**: 543-553

2.��������� Mourrain, P., Beclin, C., Elmayan, T., Feuerbach, F., Godon, C., Morel, J.-B., Jouette, D. *et al.* (2000). *Arabidopsis SGS2* and *SGS3* genes are required for posttranscriptional gene silencing and natural virus resistance. *Cell* **101**: 533-542

3.��������� Gazzani, S., Lawrenson, T., Woodward, C., Headon, D. & Sablowski, R. (2004). A link between mRNA turnover and RNA interference in Arabidopsis. *Science* **306**: 1046-1048

4.��������� Himber, C., Dunoyer, P., Moissiard, G., Ritzenthaler, C. & Voinnet, O. (2003). Transitivity-dependent and -independent cell-to-cell movement of RNA silencing. *EMBO J* **22**: 4523-4533

5.��������� Schwach, F., Vaistij, F. E., Jones, L. & Baulcombe, D. (2005). An RNA-dependent RNA-polymerase prevents meristem invasion by Potato virus X and is required for the activity but not the production of a systemic silencing signal. *Plant Physiol* **138**: 1842-1852

6.��������� Peragine, A., Yoshikawa, M., Wu, G., Albrecht, H. L. & Poething, R. S. (2004). *SGS3* and *SGS2/SDE1/RDR6* are required for juvenile development and the production of *trans-acting* siRNAs in *Arabidopsis*. *Genes Dev* **18**: 2368-2379

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Reporter genes

The products of reporter genes are easily visible or can be visualised indirectly by simple assays. Frequently used examples are the GUS gene (visualised by a blue dye) and the GFP gene. The latter, which has been used extensively in RNA silencing studies, is visualised by its distinct green fluorescence under UV light (in contrast to the red auto-fluorescence of green plant tissues. This is a non-disruptive method that can be used to follow the development of gene expression or silencing on a plant without killing it.

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rgs-CaM

Rgs-CaM is a calmodulin-related protein that has been identified as plant-encoded suppressor of post-transcriptional gene silencing. This protein interacts with a virus-encoded silencing suppressor [1] . Its exact role in the silencing machinery is still unknown.

Literature

1.��������� Anandalakshmi, R., Marathe, R., Ge, X., Herr Jr., J. M., Mau, C., Mallory, A., Pruss, G. *et al.* (2000). A Calmodulin-Related Protein That Suppresses Posttranscriptional Gene Silencing in Plants. *Science* **290**: 142-144

Ribozymes

While most chemical reactions in a living cell are catalysed by proteins, RNA can sometimes have enzymatic activity as well. Such RNA enzymes are called ribozymes. Their activity, like the enzymatic activity of proteins, is defined by their three-dimensional structure, which is a consequence of the interactions between nucleobases within the RNA strand. Ribozymes often cleave themselves or other RNAs and can be engineered to target specific messenger RNAs for destruction. This method is now largely replaced by RNA silencing techniques.

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SDE1

See RDR6

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SGS2

See RDR6

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SGS3

A protein of unknown function that is required for post-transcriptional gene silencing and antiviral defence [1] . It is also involved in the biosynthesis of trans-acting siRNA [2,3] . There are no homologues of this protein in animals [1] .

Literature

1.��������� Mourrain, P., Beclin, C., Elmayan, T., Feuerbach, F., Godon, C., Morel, J.-B., Jouette, D. *et al.* (2000). *Arabidopsis SGS2* and *SGS3* genes are required for posttranscriptional gene silencing and natural virus resistance. *Cell* **101**: 533-542

2.��������� Peragine, A., Yoshikawa, M., Wu, G., Albrecht, H. L. & Poething, R. S. (2004). *SGS3* and *SGS2/SDE1/RDR6* are required for juvenile development and the production of *trans-acting* siRNAs in *Arabidopsis*. *Genes Dev* **18**: 2368-2379

3.��������� Vazquez, F., Vaucheret, H., Rajagopalan, R., Lepers, C., Gasciolli, V., Mallory, A. C., Hilbert, J.-L. *et al.* (2004). Endogenous trans-acting siRNAs Regulate the Accumulation of Arabidopsis mRNAs. *Mol Cell* **16**: 69-79

Size classes of siRNAs

Originally thought be of uniform length, plant siRNAs were later found to occur in distinct size classes, with 21-22 and 24-25 nucleotide siRNAs being the two major fractions [1,2] . These are often referred to as short and long siRNAs respectively. The short siRNAs are associated with local silencing and the short-range signal, whereas systemic signalling and RNA-directed DNA methylation are both associated with the long size class [1,3] . In *Arabidopsis*, DCL1 has been identified as a Dicer activity that produces short siRNAs, while DCL3 produces the long size class [4] .

Literature

1.��������� Hamilton, A. J., Voinnet, O., Chappell, L. & Baulcombe, D. C. (2002). Two classes of short interfering RNA in RNA silencing. *EMBO J* **21**: 4671-4679

2.��������� Tang, G., Reinhart, B. J., Bartel, D. P. & Zamore, P. D. (2003). A biochemical framework for RNA silencing in plants. *Genes Dev* **17**: 49-63

3.��������� Himber, C., Dunoyer, P., Moissiard, G., Ritzenthaler, C. & Voinnet, O. (2003). Transitivity-dependent and -independent cell-to-cell movement of RNA silencing. *EMBO J* **22**: 4523-4533

4.��������� Qi, Y., Denli, A. M. & Hannon, G. J. (2005). Biochemical specialization within Arabidopsis RNA silencing pathways. *Mol Cell* **19**: 421-428

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Transcriptome

The sum of all transcripts (RNA) derived from the genome (DNA) in a living cell is referred to as its transcriptome.

Uridylation

In the absence of HEN1, miRNAs and siRNAs are not methylated [1,2] . Uridylation, i.e. addition of uridine nucleotides, of these unmethylated small RNAs is believed to induce their degradation [3] .

Literature

1.��������� Yu, B., Yang, Z., Li, J., Minakhina, S., Yang, M., Padgett, R. W., Steward, R. *et al.* (2005). Methylation as a Crucial Step in Plant microRNA Biogenesis. *Science* **307**: 932-935

2.��������� Li, J., Yang, Z., Yu, B., Liu, J. & Chen, X. (2005). Methylation Protects miRNAs and siRNAs from a 3'-End Uridylation Activity in Arabidopsis. *Curr Biol* **15**: 1501-1507

3.��������� Shen, B. & Goodman, H. M. (2004). Uridine Addition After MicroRNA-Directed Cleavage. *Science* **306**: 997

Viroids

Viroids are plant pathogens that are similar to viruses. They consist of single-stranded RNA that, unlike viral RNA, is not coated with proteins. Instead, it is highly structured, which may confer some resistance against RNA degrading enzymes. Viroid RNA, in contrast to viruses, does not encode any proteins and, consequently, the viroid relies completely on host proteins for replication and movement through the plant.

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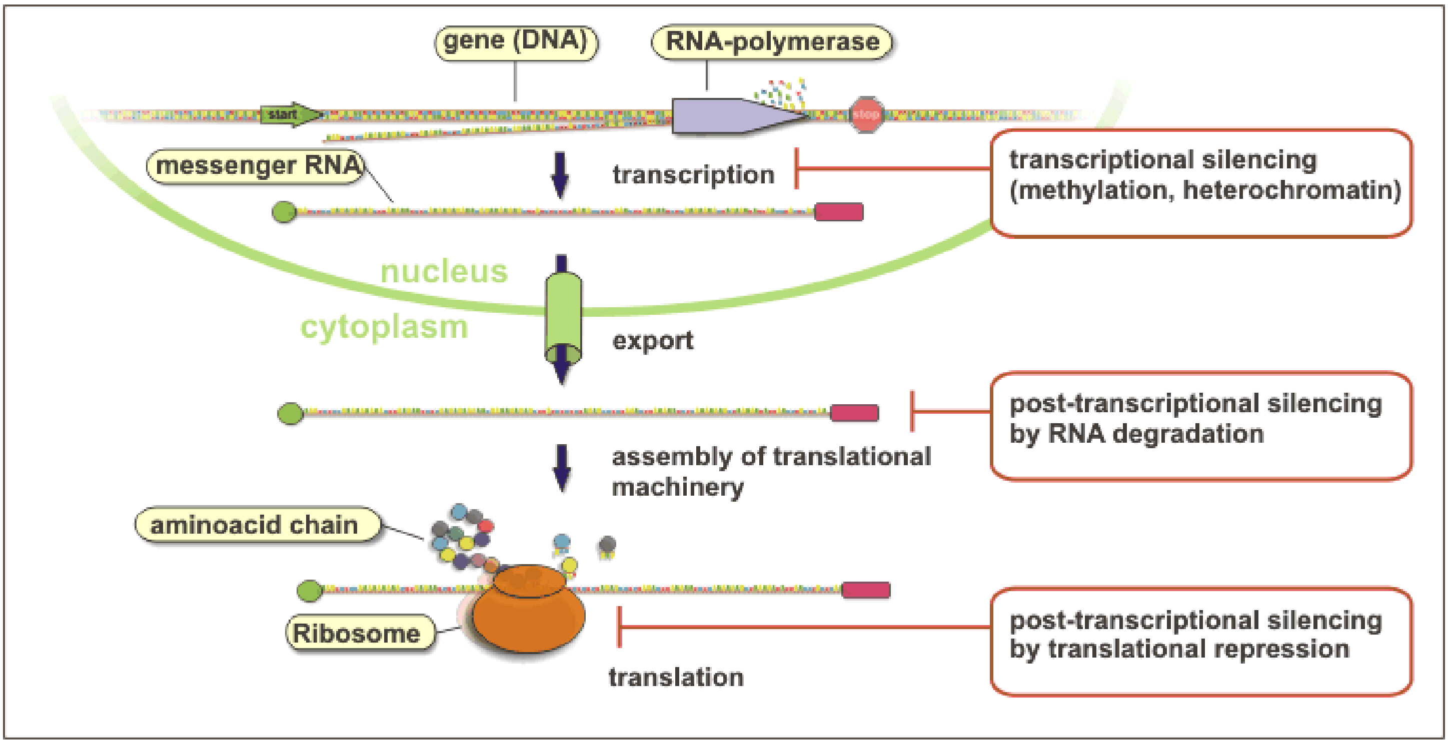
Transcription, translation and modes of gene silencing



In the nucleus, genes are transcribed by RNA-polymerases. The transcripts are processed further to yield the mature messenger RNA which is characterised by structures at both ends (5' cap and 3' poly(A) tail). After export to the cytoplasm, the translation machinery assembles on the messenger RNA and ribosomes scan the sequence, translating it into chains of aminoacids that eventually form the mature protein.

Gene silencing can interfere with this process at different stages. Transcriptional silencing affects the genomic DNA itself by introducing methylation and changes to the chromatin structure, which render the affected region inactive. In contrast, Post-transcriptional silencing affects the messenger RNA, either by destroying it or by blocking translation. All types of gene silencing are sequence specific, thus only genes and messenger RNAs with sufficient sequence similarity to the original trigger are affected.

Most applications of RNA silencing in GM plants employ post-transcriptional mechanisms.

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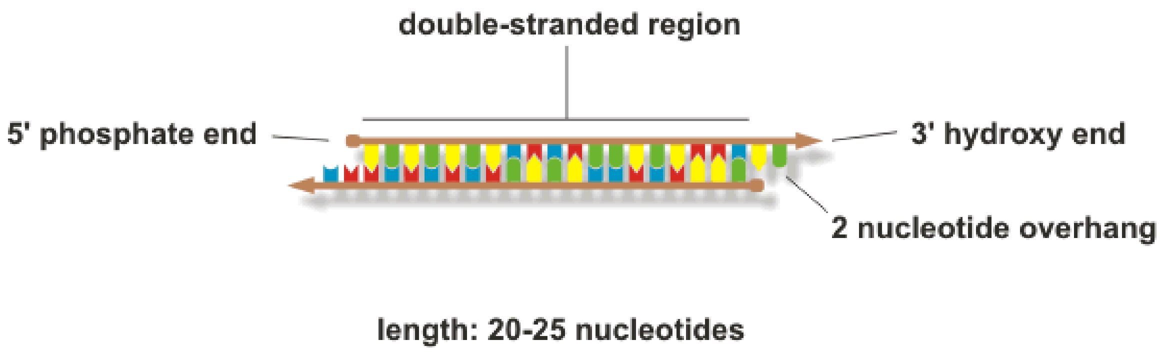
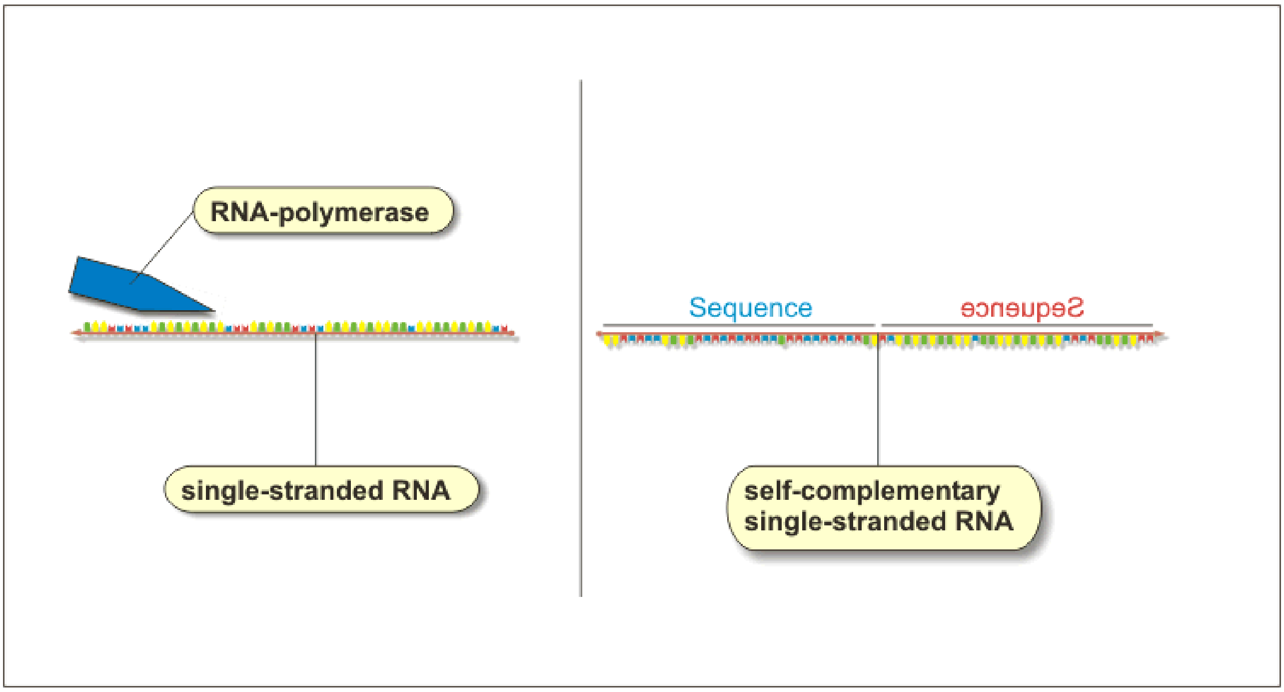
siRNA - basic structure



Small interfering (si)RNAs are double-stranded RNA molecules, 20-25 nucleotides in length, with two unpaired bases at the 3' ends of each strand. They are the mediators of sequence specificity in RNA silencing.

Post-transcriptional RNA silencing triggered by single-stranded RNA (series of 9 slides)



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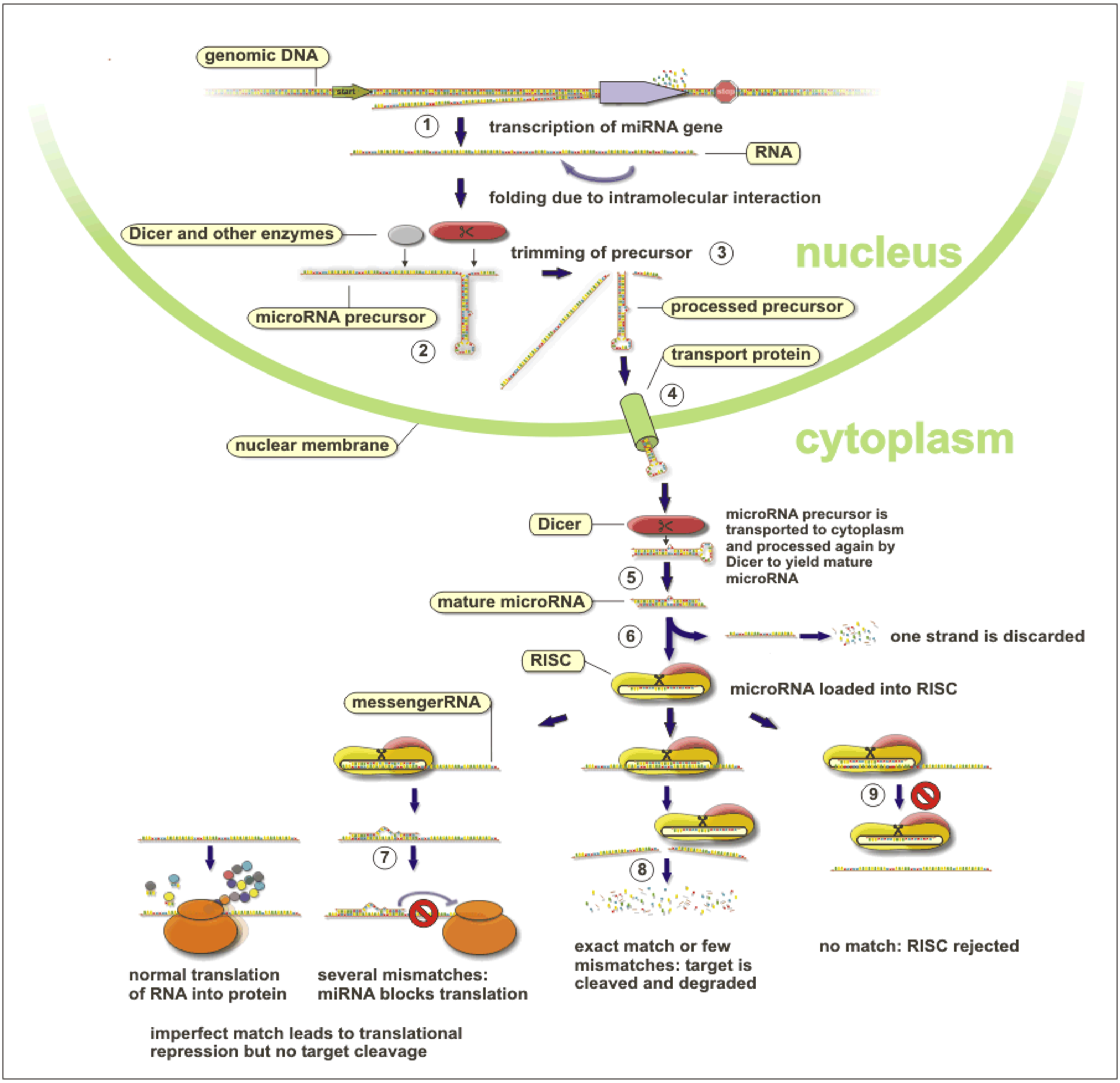
Step 1 of 9

This series of images shows how single-stranded RNAs can trigger RNA silencing.
A single-stranded RNA (left) can be recognised by an RNA-dependent RNA polymerase, e.g. if it exhibits aberrant features such as missing end structures. The polymerase then synthesises the second (complementary) strand .
Some RNAs are designed to form double-strands (right) because one part is complementary to the other (see here for details). The nucleobases are shown here as coloured flags where yellow can pair with red and blue with green (see next slide).

Overview of the micro (mi)RNA pathway



This illustration gives an overview of the micro (mi)RNA silencing pathway

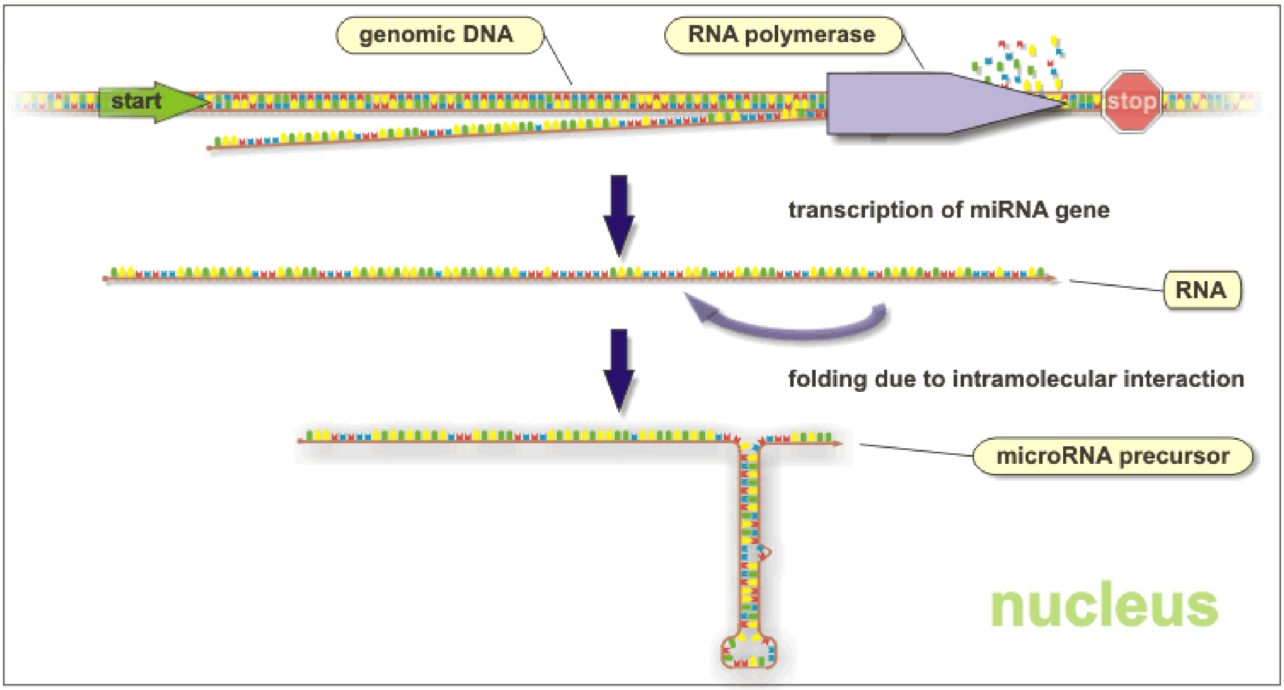
MicroRNAs regulate the expression of genes by silencing messenger RNAs. The miRNA gene is transcribed (1) into a precursor that folds into a characteristic structure with partially double-stranded regions (2). In plants, the miRNA precursor is then trimmed by a Dicer enzyme and, presumably, other enzymes (3). The shorter precursor is transported from the nucleus to the cytoplasm (4) where it is processed by Dicer to yield the mature miRNA (5).

Only one strand of the miRNA is selectively incorporated into the silencing effector complex RISC while the other strand is degraded (6). Once programmed with a miRNA, RISC can interact with messenger RNAs in three different ways:

- an imperfectly matched miRNA can bind to its target but does not induce its destruction. The bound miRNA blocks the translation of the messenger RNA into protein (7). This is the default mode of action for most animal miRNAs but it is rare in plants.
- a perfectly matched miRNA or a miRNA with a small number of mismatches (usually not more than 3 in plants) induces target cleavage and destruction (8).
- a poorly matched miRNA has no affect on the messenger RNA (9).

A more detailed step by step explanation can be found here.

Micro (mi)RNA guided RNA silencing (series of 5 slides)



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Step 1 of 5

This series of images shows how micro (mi)RNAs are produced and how they induce silencing in plants.

MicroRNAs are endogenous small RNAs, i.e. they are derived from the plant's own genome. The miRNA biogenesis pathway starts in the nucleus of the cell with the transcription of the gene that encodes the miRNA precursor. This is an RNA that, in contrast to messenger RNAs, does not code for a protein. Instead, the precursor RNA folds into a characteristic structure by intramolecular base-pairing. This structure contains double-stranded regions where most nucleobases establish a basepair.

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