EXPLORING PLANT GENOMES BY RNA-INDUCED GENE SILENCING

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The nucleotide sequences of several animal, plant and bacterial genomes are now known, but the functions of many of the proteins that they are predicted to encode remain unclear. RNA interference is a gene-silencing technology that is being used successfully to investigate gene function in several organisms — for example, *Caenorhabditis elegans*. We discuss here that RNA-induced gene silencing approaches are also likely to be effective for investigating plant gene function in a high-throughput, genome-wide manner.

CO-SUPPRESSION
The silencing of an endogenous gene due to the presence of a homologous transgene or virus. Co-suppression can occur at the transcriptional or post-transcriptional level.

AGROBACTERIUM TUMEFACIENS
A gram-negative soil bacterium that is used to transfer DNA into plant cells by a process similar to bacterial conjugation. The transferred DNA (T-DNA) randomly integrates into the plant genome to produce stably transformed plants.

The genomes of several animal species and two plant species, *Arabidopsis thaliana*[1] and *Oryza sativa* (rice)[2,3], have been sequenced. Analyses of sequence data from these two plant species indicate that there might be ~25,000 protein-encoding genes in *Arabidopsis* and up to 55,000 in rice. A key challenge is now to identify how each of these genes functions in the growth and development of these plants.

Reverse genetics, in which a gene is disrupted so that the effect (if any) of its loss on an organism can be observed, is a simple way to investigate gene function. A reverse-genetic approach called insertional mutagenesis has been a key in studying *Arabidopsis*. Two insertional mutagenesis strategies — one based on transferred DNA (T-DNA)[4], the other on transposon tagging[5,6] — have been developed in this plant and have given rise to several large, publicly available collections of *Arabidopsis* insertion mutants (such as the *Arabidopsis* Stock Centre and the Salk Collection at The *Arabidopsis* Information Resource (TAIR)). Although these collections have provided the plant research community with a superb resource of null mutants, the approach of using insertion mutants has several limitations. For example, it cannot be used to investigate the functions of duplicated genes, and many mutant phenotypes in these lines are caused by disruptions to genes other than those into which the DNA tag is inserted. Antisense and CO-SUPPRESSION[7] technologies have also been useful tools for investigating gene function in plants, but they are labour intensive and somewhat unpredictable[8].

One approach that can circumvent these limitations is RNA-induced gene silencing. It is variously termed post-transcriptional gene silencing (PTGS) in plants, quelling in fungi and RNA interference (RNAi) in animals[9–18]. The essence of RNA-induced gene-silencing is the delivery of double-stranded RNA (dsRNA) into an organism, or cell, to induce a sequence-specific RNA degradation mechanism that effectively silences a targeted gene. How RNAi operates and its natural role for virus defence and endogenous gene regulation in plants have been reviewed elsewhere[8,19–23], and so are not the focus of this review. Instead, we discuss and compare the various ways in which RNAi can be carried out in plants and assess how effective each technology is for high-throughput, genome-wide studies of plant gene function. Although we use the term RNAi, in place of PTGS, to describe this process in plants, the use of RNAi to describe RNA-induced gene silencing in plants remains a matter of debate.

Inducing RNA interference in plants

When naturally occurring viral RNA (that produces dsRNA during its replication), self-complementary, single-stranded 'hairpin' RNA (hpRNA) or dsRNA, is introduced into a plant, it is degraded into ~21-nucleotide dsRNA fragments, known as small interfering RNAs (siRNAs). These siRNA fragments are then incorporated into a nuclease-containing complex called RISC (RNAi silencing complex), which degrades mRNAs that are complementary to the single-stranded siRNA that is associated with the complex[23] (FIG. 1).

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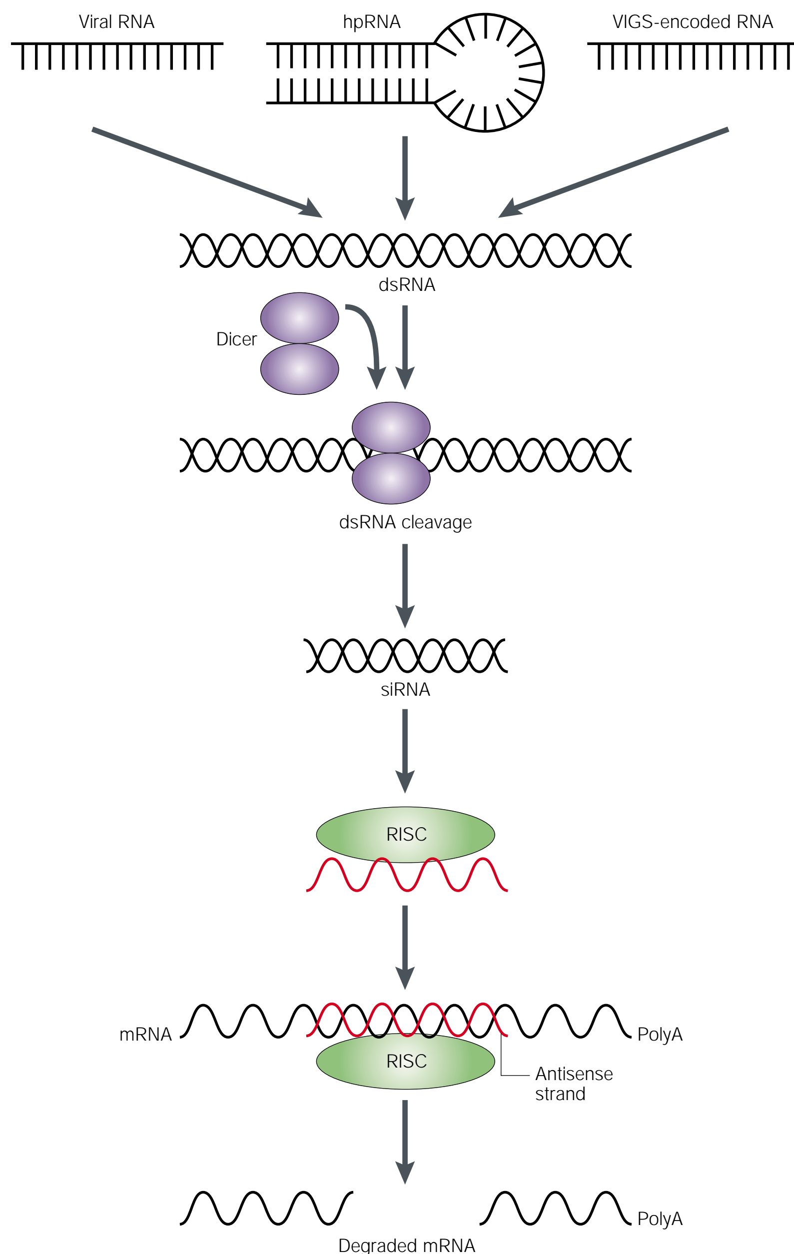

Figure 1 | **The current model of RNA-mediated gene silencing in plants.** This model is based on the results of *in vitro* studies of RNA-induced gene silencing, or RNA interference (RNAi), in animal extracts (reviewed in REF. 23). RNAi is believed to operate in a similar manner in plants because small interfering RNAs (siRNAs) are found in silenced plants, and plants have homologues of the animal gene *Dicer*. Double-stranded RNA (dsRNA) from replicating viral RNA, viral-vector-derived (VIGS, or virus-induced gene silencing) RNA or hairpin RNA (hpRNA) transcribed from a transgene, is processed by a Dicer-containing complex to generate siRNAs. An endonuclease-containing complex (called the RNAi silencing complex, RISC), is guided by the antisense strand of the siRNA to cleave specific mRNAs, so promoting their degradation.

MONOCOTYLEDON (monocot). One of the two classes of flowering plants that is characterized by one embryonic leaf (cotyledon). Maize, rice and other grasses are common monocots.

An important aspect of using RNAi in plant genomics research is the delivery of the silence-inducing dsRNA or hpRNA. This RNA can be delivered by stably transforming plants with transgenes that encode hpRNAs or viral RNAs. It can also be transiently delivered by bombarding plants with nucleic-acid-coated beads, by infiltrating plant cells with transgene-carrying *AGROBACTERIUM TUMEFACIENS* or by infecting plants with a virus, either on its own or together with a satellite virus. Each delivery method has its own advantages and disadvantages (see below).

Microprojectile bombardment and agroinfiltration. The bombardment of plant tissues with gold or tungsten particles that have been coated with DNA is an approach that has been used routinely for studying gene expression in plants and for transformation of several MONOCOTYLEDON (monocot) plant species, such as rice[24]. The DNA or RNA that coats each particle is released and expressed in the cells where the particles come to rest. This procedure has also recently been used to induce RNAi in plants by delivering dsRNA or DNA constructs that encode hpRNA into leaf epidermal cells of the cereal species, maize, wheat and barley[25]. The bombardment of monocot leaves with *in vitro* transcribed dsRNA of *A1* (a gene from the anthocyanin biosynthetic pathway), *MLO* (a negative regulator of the resistance to barley powdery mildew) or β-GLUCURONIDASE (*GUS*) sequences[25], resulted in the reduced activity of each of these target genes. RNAi has also been induced against a *GFP* (green fluorescent protein) transgene in *Nicotiana benthamiana*. In this study, *N. benthamiana* plants were bombarded with particles coated with one of several types of silence-inducing molecules: dsRNA; siRNA; a plasmid DNA that encoded single-stranded *GFP* RNA[26]; and dsDNA that targeted the transgene[27]. In each case, the visually scorable phenotype of suppressed GFP fluorescence was observed. In another study, a repressor of *GUS* in a transgenic tobacco plant (*Nicotiana tabacum*) was silenced by bombarding the plant with dsRNA and siRNA, and with sense or antisense RNAs. Each approach induced the production of siRNAs that silenced the repressor, resulting in *GUS* expression[26].

When *Agrobacterium tumefaciens* infects a plant, it transfers part of its T-DNA plasmid into the genome of the infected plant cells. The genes on the T-DNA seem to be expressed in the plant cell both during the transfer process and after the T-DNA has integrated into the plant genome. Infiltrating leaves[28] (using a needle-less syringe to pass liquid through the STOMATA into the intercellular spaces) with a culture of *Agrobacterium*, in which the T-DNA plasmid contains a transgene that encodes an endogenous plant gene sequence, can trigger RNAi against the target endogenous gene, although it is not clear how this single, sense transcript generates dsRNA. Nevertheless, RNAi by this method has been shown in transgenic *N. benthamiana* plants that express *GFP*[29,30]. In the first days after infiltration, *GFP* is overexpressed at the site of infiltration, but after about three days this expression subsides to undetectable levels, concomitant with a reduction of endogenous *GFP* expression. This localized silencing subsequently spreads throughout the plant. Similar, but more potent, silencing has been found to occur when T-DNAs that contain hpRNA-encoding sequences are used for agroinfiltration[30].

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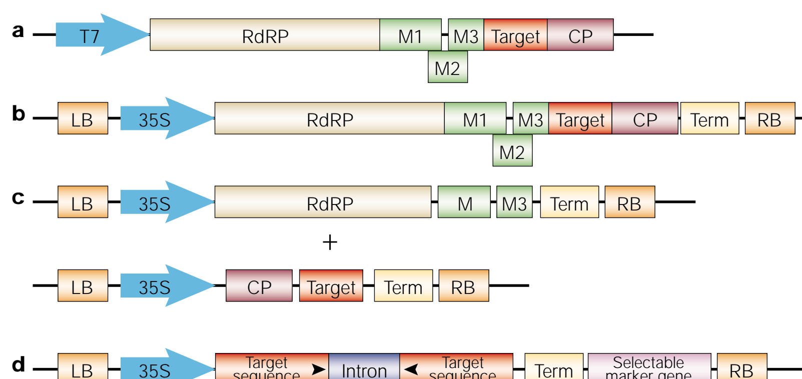

Figure 2 | **DNA constructs for RNA-mediated gene silencing. a** | A DNA plasmid that can be propagated in *Escherichia coli* from which infectious potato virus X (PVX) RNA can be transcribed *in vitro*, using T7 polymerase. The PVX cassette contains sequence derived from the gene to be targeted. **b** | A transferred (T)-DNA plasmid that is propagated in *Agrobacterium*. When this plasmid-carrying *Agrobacterium* is inoculated onto a plant, it transfers the DNA between its left (LB) and right (RB) borders into the plant's cells. The region between the borders contains the viral sequences shown in part **a**, but in this vector, the T7 promoter has been replaced with the cauliflower mosaic virus promoter. This enables the transferred DNA to be transcribed by the plant's endogenous transcription machinery to generate infectious PVX (plus insert sequence) RNA. In amplicon transgene vectors, a selectable maker gene is also present between the left and right borders of this plasmid, enabling plants to be stably transformed with the transferred DNA. **c** | The tobacco rattle virus (TRV) virus-induced gene-silencing (VIGS) system. Two T-DNA plasmids that encode the TRV genome (one encoding TRV RNA1 and the other encoding TRV RNA2, which carries the inserted target sequence) are propagated separately in *Agrobacterium* and used to co-infect plant tissue. **d** | A typical T-DNA plasmid for the expression of hairpin RNAs (hpRNAs). This plasmid can be transiently introduced into plants by bombardment or stably introduced by agroinfiltration. A generic silencing precursor construct (pHANNIBAL) that enables hpRNA vectors to be easily constructed has different multiple cloning sites either side of the intron to enable the rapid insertion of target sequences in forward and reverse orientations. 35S, CaMV 35S promoter; CP, coat protein; M1,2,3, movement proteins 1, 2, 3; RdRP, RNA-dependent RNA polymerase; T7, T7 promoter; Term, transcription termination sequence.

β-GLUCURONIDASE
(GUS). An easily visualized reporter gene that is used in plant research.

STOMATA
Natural openings in the epidermis of a stem or leaf of a plant that are surrounded by specialized guard cells, and allow gas exchange with the air.

CAMV 35S PROMOTER
A promoter derived from cauliflower mosaic virus that has been widely used in transgenic plants because of its ability to direct high-level constitutive transcription.

GEMINIVIRUSES
A specific group of viruses that have genomes composed of single-stranded DNA.

MERISTEM
The undifferentiated tissue at the tips of stems and roots in which new cell division is concentrated.

Virus-induced gene silencing. Most plant viruses have single-stranded RNA genomes, which are released from the protein coat of their virus particles as they enter a cell. Their genomic RNA is then replicated by the virus-encoded RNA-dependent RNA polymerase to produce sense and antisense RNA. These viral RNAs (which have the potential to hybridize to form dsRNA) trigger an RNAi response against their sequences[31–33] (FIG. 1).

Viruses have several features that make them particularly useful to plant researchers. The naked RNAs — that is, viral RNAs without the protection of a virus particle — of several plant viruses can be applied to plants to cause infection. These RNAs can be generated by *in vitro* transcription of a cDNA clone that encodes a complete virus sequence (FIG. 2a). Similarly, a full-length virus cDNA clone that has been placed into a T-DNA plasmid, expressed under the control of a cauliflower mosaic virus (CAMV) 35S PROMOTER (FIG. 2b,c) and delivered to plants by agroinfiltration, can produce viral infection when it is transcribed by the plant[34].

It is also possible to introduce exogenous sequences into specific locations in the genome of a virus, and retain the infectivity of the RNA transcript. When these transcripts are used to infect plants, the foreign sequences also induce, and become the target of, the RNAi response of the host plant. This ability of viruses to carry and induce RNAi against foreign sequences has been harnessed into a technology referred to as virus-induced gene silencing (VIGS)[33].

The first demonstration of VIGS by Monto Kumagai and colleagues[35] came well before the concept of RNAi had been developed. A fragment of an endogenous plant gene, phytoene desaturase (*PDS*), which encodes an enzyme of the carotenoid biosynthesis pathway, was inserted into an infectious clone of tobacco mosaic virus (TMV), so that the virus would produce either a sense or an antisense *PDS*-derived RNA. The tobacco plants infected with either modified virus showed marked photobleaching. This result showed that the endogenous *PDS* gene of the plant was silenced because tissues that lack carotenoids photobleach under strong light conditions. David Baulcombe's laboratory has developed robust and refined VIGS systems that use potato virus X (PVX)[32] and tobacco rattle virus (TRV)[34]. There are now infectious clones of several plant viruses that have been used as VIGS vectors[32–46], most of which have RNA genomes. However, some DNA viruses (mainly the GEMINIVIRUSES), which possibly produce dsRNA by transcriptional readthrough beyond their terminators, have also been found to induce RNAi[39–42].

Usually, 300–800-nucleotide fragments of target gene sequences are used in VIGS systems (TABLE 1), but sequences as short as 23–60 nucleotides can be effective[37,47]. Many of the different VIGS vectors have been tested against the reporter genes *GUS* or *GFP*, and the endogenous gene *PDS*. TRV–VIGS has also been used against a range of endogenous genes, especially in *N. benthamiana*, and has been particularly useful for identifying genes involved in disease resistance and ethylene signalling (TABLE 1). PVX, TMV and many other viruses do not infect plant MERISTEMS, but TRV seems to infect almost all tissues, including meristems and floral organs. The widespread distribution of TRV throughout the plant probably makes TRV–VIGS[34,43–46] the better system.

The TRV genome comprises two RNAs: RNA1, which encodes several genes, including the RNA-dependent RNA polymerase; and RNA2, which encodes the coat protein. The target gene sequence is inserted into RNA2, downstream of the gene that encodes the coat protein, and this modified RNA is co-inoculated with unmodified RNA1 onto a plant to generate an infection and induce VIGS. These viral RNAs can be made by transcribing *in vitro* the appropriate DNA constructs, but an easier way is to use the TRV–AgroVIGS constructs (FIG. 2c). In this system[44], a T-DNA plasmid that contains the CaMV 35S promoter and encodes RNA1 is propagated in one culture of *Agrobacterium*, and a similar construct that encodes RNA2 (and the target sequence) is propagated in a different *Agrobacterium* culture. An aliquot of each culture is mixed together and infiltrated into plants to initiate infection and RNAi.

Some geminivirus–VIGS systems also seem to be promising tools for genomic research. The tomato golden mosaic geminivirus-based VIGS system is an effective initiator of targeted RNAi[39] in *N. benthamiana*, and has been shown to be capable of silencing the expression of the endogenous proliferating cell nuclear

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SATELLITE VIRUS RNA
A specific parasitic RNA that depends on a virus for its replication.

Table 1 | **Examples of endogenous plant genes silenced by VIGS and their phenotypes**

Gene	Species	Virus vector	Target size (nt)	Phenotype	References
PDS	Tobacco *N. benth* Tomato Barley	Various	369 409 409 185	Photobleaching	35 34 44 38
CESA	*N. benth*	PVX	377	Distended cell walls	36
EDS1	*N. benth*	TRV	548	Compromised *N*-gene hypersensitive response*, leading to susceptibility to TMV.	43,45
RAR1	*N. benth*	TRV	468	Same as for *EDS1*	45
NPR1	*N. benth*	TRV	753	Same as for *EDS1*	45
SGT1	*N. benth*	TRV	479	Same as for *EDS1*	46
SKP1	*N. benth*	TRV	411	Same as for *EDS1*	46
CSN3	*N. benth*	TRV	539	Same as for *EDS1*	46
CSN8	*N. benth*	TRV	298	Same as for *EDS1*	46
NPK1	*N. benth*	TRV	110	Same as for *EDS1*. *NPK1*-silenced plants also showed defective *Bs2* and *Rx* gene-mediated pathogen resistance, defective cytokinesis, reduced cell size and an overall dwarf phenotype	47
#3	*N. benth*	TRV	872	Same as for *EDS1*	71
#13	*N. benth*	TRV	420	Increased *N*-gene resistance to TMV	71
CDPK2	*N. benth*	TRV	417	Delayed hypersensitive response	72
RBCS	*N. benth* and tomato	TRV	500	Pale yellow leaves	44
CTR1	Tomato	TRV	690	Ethylene-related dwarfism	44

*The *N*-gene is an NBS–LRR (nucleotide-binding-site–leucine-rich-repeat)-type resistance gene, which causes localized cell death at sites of infection by tobacco mosaic virus (TMV). *CESA*, cellulose synthase A; *CPDK2*, calcium-dependent protein kinase 2; *CTR1*, constitutive triple response 1; *EDS1*, *RAR1*, *NPR1*, *SGT1*, *SKP1*, *CSN1*, *CSN8*, *NPK1*, *#3* and *#13* are all *N*-gene response-pathway genes; *N. benth*, *Nicotiana benthamiana*; nt, nucleotides; *PDS*, phytoene desaturase; PVX, potato virus X; *RBCS*, rubisco small subunit; TRV, tobacco rattle virus.

antigen (*PCNA*) gene in meristem tissues[41]. The cabbage leaf curl geminivirus (CbLCV) has the potential to be even more useful, because a VIGS system based on this virus has recently been shown to infect and induce RNAi in *Arabidopsis*[42].

A new viral silencing system, using SATELLITE RNAS, has also been recently developed. In this satellite-virus-induced silencing system (SVISS)[48], the target sequence is inserted into the satellite RNA, which is then co-inoculated with the associated virus. SVISS has been shown to work with more than ten genes, including *PDS*, plastid transketolase and *CESA* (cellulose synthase A), using a satellite of the U2 strain of TMV in tobacco[48] (FIG. 3).

Amplicon and hairpin RNA transgenes. Amplicon and hpRNA transgenes are stably transformed in plants, and they ensure that the RNAi they induce is inherited by subsequent generations. An amplicon transgene encodes a virus-derived transcript that contains a target gene sequence, but not necessarily that of all the genes of the native virus, and can direct its own replication. The transgene is usually under the control of the CaMV 35S promoter (FIG. 2), so that the amplicon RNA is expressed in almost every cell of the plant, although the transfected plants do not show symptoms of being infected with virus[49]. Amplicon constructs have not been as widely used as VIGS. They have been based mainly on PVX and have been used

Figure 3 | **Tobacco plant phenotypes after infection with a satellite-virus-induced silencing system.** Results show plant phenotypes four weeks after infection. Phenotypes caused by the silencing of the genes that encode **a** | cellulose synthase, **b** | transketolase and **c** | phytoene desaturase are shown. Images courtesy of M. Metzlaff, Ghent, Belgium. Reproduced with permission from REF. 48 © (2002) Blackwell Publishing.

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Table 2 | **Examples of endogenous plant genes silenced by hpRNAs and their phenotypes**

Gene	Species	Target size (nt)	Phenotype	References
PPO	Tobacco	572	Reduced oxidation	54
PDS	*Arabidopsis*	430	Photobleaching	54
EIN2	*Arabidopsis*	600	Ethylene insensitivity	54
FLC1	*Arabidopsis*	400	Early flowering	54
CHS	*Arabidopsis*	741	Reduced pigment	54
AG	*Arabidopsis*	554	Mutant flowering structure phenotypes	55
CLV3	*Arabidopsis*	288	Mutant flowering structure phenotypes	55
AP1	*Arabidopsis*	409	Mutant flowering structure phenotypes	55
PAN	*Arabidopsis*	339	Mutant flowering structure phenotypes	55
CBL	*Arabidopsis*	1146	Altered biosynthesis	56
SAD1	Cotton	514	Altered composition of seed oil	54
FAD2	*Arabidopsis* and cotton	98–853	Altered composition of seed oil	54,57,73

AG, agamous; *AP1*, apetala 1; *CBL*, cystathionine β-lyase; *CHS*, chalcone synthase; *CLV3*, clavata 3; *EIN2*, ethylene signalling 2; *FAD2*, fatty acid Δ12-desaturase; *FLC1*, flowering repression 1; nt, nucleotides; *PAN*, perianthia; *PDS*, phytoene desaturase; *PPO*, polyphenol oxidase; *SAD1*, stearoyl-ACP Δ9-desaturase.

successfully to silence the reporter transgenes, *GUS*[49] and *GFP*, and the endogenous genes *DWARF*, *RUBISCO* and *PDS*[50] in tobacco, *N. benthamiana* and tomato. An amplicon construct based on tobacco yellow dwarf geminivirus has also been used to silence chalcone synthase in petunia[51]. Interestingly, a *PVX–GFP* amplicon transgene induced *GFP*-specific RNAi in *Arabidopsis*[52], even though *Arabidopsis* is not a host of PVX. This indicates that amplicons might overcome the host specificities that restrict the use of VIGS to the host range of the virus.

An alternative method of inducing RNAi in plants is to use transgenes that express a self-complementary hpRNA. A hpRNA forms when an RNA hybridizes with itself to form a hairpin structure that comprises a single-stranded loop region and a base-paired stem, which mimics dsRNA and provides the specificity of the RNAi. (The loop plays no part in directing RNAi.) The frequency with which RNAi can be effectively induced in transformed plants can be increased by using hpRNA transgenes in which the loop size is minimized by using an intron[52,53] (FIG. 2d). The induction of RNAi by a transgene that encodes an hpRNA was first shown by using the *GUS* reporter gene in rice[12]. In this experiment, the construct was under the control of the strong and constitutive maize ubiquitin promoter. So far, most hpRNA constructs have been used in DICOTYLEDONS and have been expressed under the control of the strong, constitutive CaMV 35S promoter[53–56]. Indeed, a generic vector, pHANNIBAL[54], incorporating the CaMV 35S promoter, has been created, which enables hpRNA constructs to be easily generated (FIG. 2d). However, seed-specific promoters, such as the napin and lectin promoters[53,54,57] have also been effective at silencing seed-expressed genes. A wide variety of genes, ranging from transcription factors to metabolic biosynthesis enzymes, as well as viral sequences, have been effectively silenced using hpRNA transgenes (TABLE 2), and many of the phenotypes obtained have been similar to those of counterpart insertion mutants. As with VIGS, most of the gene fragments that have been encoded by hpRNA vectors have been between 300 and 800 nucleotides, although a fragment as small as 98 bases has been effective[54]. Results with hpRNAs indicate that 5′ or 3′ untranslated regions or the coding region of an mRNA might all be good silencing targets[54].

DICOTYLEDON
(dicot). One of the two principal classes of flowering plant that is characterized by two cotyledons (primitive leaves) in the embryonic plant. Tomatoes, maple trees and mustard are common dicots.

Transcriptional gene silencing

A recently discovered feature of RNAi in plants is that dsRNA induces and directs not only sequence-specific RNA degradation, but also sequence-specific DNA methylation[58–60]. The VIGS or hpRNA constructs described so far in this review use sequences contained in the mRNA of target genes to bring about silencing. This often results in the concomitant methylation of the coding region of the target gene, which usually has no direct effect on its transcription. However, in three RNAi studies in plants, a gene promoter has been targeted, resulting in promoter methylation. This promoter methylation, or changes in chromatin conformation as a result of methylation at the promoter, presumably affect the binding of transcription factors, leading to reduced transcription (FIG. 4). In these experiments, hpRNAs were targeted against transgene promoters[58,61] or against an endogenous promoter that controls the expression of dihydrofolate reductase[61]. In another study, a viral vector was used to target the CaMV 35S promoter of a transgene[62]. This process of hpRNA-directed transcriptional gene silencing might become a useful weapon in the armoury of plant genome research.

Comparison of silencing technologies

Some of the advantages and disadvantages of transient and stably integrated gene-silencing systems in plants are compared in TABLE 3. Microprojectile bombardment and non-viral agroinfiltration induction of RNAi have the potential to be valuable tools for rapidly identifying gene functions where cellular, rather than whole-plant, assays can be used. Their main disadvantages are that

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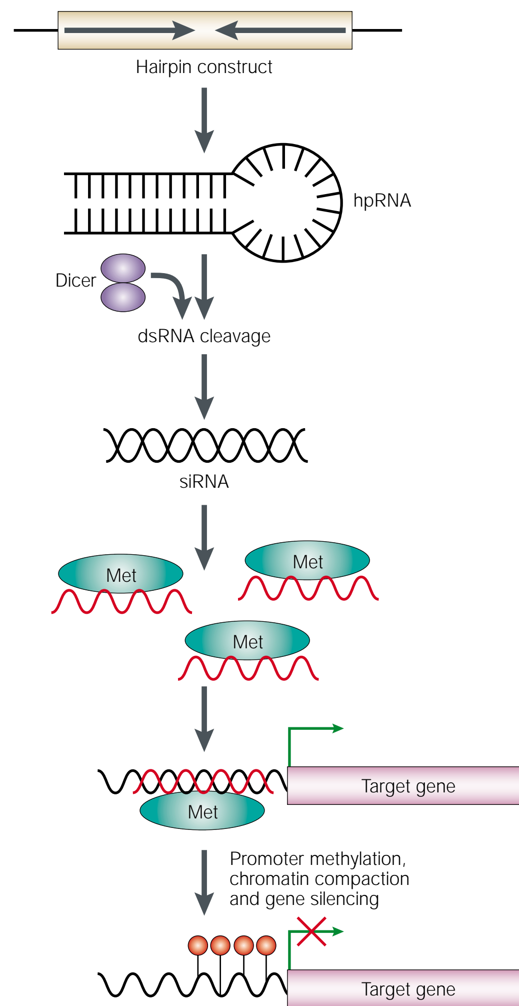

Figure 4 | **The current model of hairpin-RNA-directed transcriptional gene silencing in plants.** Hairpin RNA (hpRNA) directed against promoter sequences gives rise to the methylation of cytosine residues (shown as red circles) at the targeted DNA sequence. This methylation directly, or indirectly, causes changes in the conformation of local chromatin, resulting in gene silencing by loss of transcription. dsRNA, double-stranded RNA; Met, *de novo* methyltransferase; siRNA, small interfering RNA.

the silencing they induce usually only persists for a few days and, as yet, non-viral agroinfiltration-mediated silencing has only been shown in *N. benthamiana* and tobacco.

So far, there are only a handful of examples in which amplicon-mediated RNAi and directed transcriptional gene silencing (TGS) have been used to silence genes, making it difficult to assess their potential impact on plant genomic research. The rules for TGS are far less resolved than those for RNAi. For example, answers are needed about which, and what size, promoter regions to target, and whether the methylation of specific cytosines or the alteration of the local chromatin structure by the methylation of some cytosines in the region, is sufficient to inhibit transcription. Amplicons share with hpRNA-encoding transgenes the advantages and disadvantages of delivery from stably inherited transgenes, and they share with VIGS the advantages and disadvantages of being induced by a modified replicating virus (see below).

At present, the two most widely used systems are VIGS in *N. benthamiana* and hpRNA transgenes in *Arabidopsis*. Clearly, both systems effectively and efficiently produce phenotypes (TABLES 1,2) in plants, so providing insights into the functions of the target genes. Transformation of plants with hpRNA constructs gives stable silencing that is inherited from generation to generation, thereby enabling the continued study of a phenotype. For example, the silencing of the *Arabidopsis* fatty acid desaturase 2 (*FAD2*) gene by a hpRNA has been maintained for five generations and has been found to be consistent in its effectiveness[57]. VIGS is particularly useful in plant species that are difficult or impossible to transform (if there is an VIGS system available for the species) or when analysing genes that are essential, either for housekeeping functions or for embryonic development (which cause embryonic lethality when knocked out), as the infectious transcripts can be applied to mature plants. VIGS is also well suited to the high-throughput analysis of genes, as VIGS vectors can be delivered by simply rubbing an infectious transcript onto a plant, although the cost of transcribing each construct *in vitro* might be prohibitively expensive on a large scale. The development of Agro-VIGS circumvents this problem because, in this approach, *Agrobacterium* cultures of the VIGS constructs can be inexpensively grown and injected[43–46].

Most viruses used for VIGS have a limited number of hosts, and the virus–host combination seems to be a crucial factor in determining the efficacy of silencing. Some of the viruses used in VIGS can cause symptoms that might mask the phenotype caused by the silencing of the target gene. Moreover, many viruses do not infect the growing points or floral parts of plants, especially the seed, precluding gene silencing in these tissues. TRV–VIGS in *N. benthamiana* overcomes many of these problems. TRV infects almost all tissues of the plant and produces mild symptoms. However, *N. benthamiana* seems to be a special plant as it somehow enables several viruses to maintain their foreign inserts and to disperse and cause silencing throughout the plant. By contrast — for example, in inoculated tobacco leaves — TMV[45] and PVX vectors that contain *GFP* do not spread from these leaves unless they recombine out the GFP sequence. However, the same chimeric viruses spread rapidly and express GFP throughout *N. benthamiana* plants.

Where it has been measured, TRV–VIGS reduces the target mRNA level by 80–90% (REFS 44–46). By contrast, transforming plants with hpRNA constructs typically generates a series of independent lines that have different phenotypes and degrees of target mRNA reduction[63]. In many hpRNA experiments, levels of the targeted mRNA have ranged from wild type to undetectable[57,63]. In experiments in which genes have been targeted with intron-containing hpRNA constructs, silencing usually causes easily visible or measurable

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Table 3 | **Advantages/disadvantages of transient- and stable-integration gene-silencing systems**

Expression method	Introduction	Advantages	Disadvantages
Transient			
	Microprojectile bombardment	Rapid; valuable for single-cell assays; wide species range	Limited to cells on leaf surface; silencing does not persist
	Agrobacterium infiltration	Rapid; easy to apply; high throughput; low cost	Untested on most species
	Viral-induced gene silencing (VIGS)	Rapid; easy to use; high-throughput vectors; can be applied to mature plants; good for species that are difficult or impossible to transform; useful in genetically intractable species	Host range limitations; might have restricted regions of silencing; depends on availability of infectious clones; viral symptoms might be superimposed on silencing phenotype; might have size restriction on insert
Stable			
	Amplicons	Extended host range; heritable; choice of tissues; no viral symptoms; tissue specificity controllable by promoter	Might have size restriction on insert; need efficient transformation technique
	Hairpin transgenes	Not restricted by host range; heritable; controllable tissue specificity; range of degrees of silencing; high-throughput vectors	Need efficient transformation technique

effects in 70–100% of the resulting plants[54,63]. For example, each of 31 independent plants transformed with an intron-containing hpRNA construct against flowering locus C (*FLC*) flowered earlier than wild-type plants[54]. FLC represses flowering and provides an easily measurable phenotype; the shorter the period between germination and flowering, the more profound the silencing. These lines showed a range of flowering times, indicating that each line has a different degree of silencing. A similar range of silencing has also been seen in hpRNA constructs targeted against the *Arabidopsis* genes: hpRNA silencing of *PDS* (FIG. 5) and of *FAD2* causes photobleaching and the production of a range of seed oil profiles, respectively[57]. In such cases, 10–20% of the independent transformants had phenotypes that were indistinguishable from those of the corresponding null mutation. This heritable range of degrees of silencing in hpRNA plants is a useful feature that can give a range of phenotypic effects that might provide new insights into gene function.

Future prospects

Researchers using *Caenorhabditis elegans* as a model system have led the way in using RNAi as a tool to test gene function on a genome-wide scale[64–66]. This has been feasible largely because of the ease with which RNAi can be delivered to worms, by either soaking them in dsRNA[65] or feeding them with bacteria that express dsRNA[66].

In plants, most of the published work on RNAi has been devoted to describing studies that investigated its mechanism, the development of RNAi delivery systems or its use to validate the already known or inferred functions of genes. However, genome-wide gene-function and gene-discovery studies using VIGS[43,44] and hpRNA transgenes are under way (BOX 1). The pHELLSGATE[54,63] and the pTRV–attP/R[44] vector series (BOX 1), which use the rapid recombination-based Gateway™ technology (see link to Gateway Technology), promise to facilitate the high-throughput cloning and manipulation of gene libraries that is required for creating RNAi constructs. Compared with conventional restriction enzyme

Figure 5 | **Degrees of silencing produced by hairpin-RNA-encoding transgenes.** The stable transformation of *Arabidopsis* plants with the same hairpin RNA (hpRNA) construct that is targeted against phytoene desaturase gives rise to lines that show a heritable photobleaching phenotype in: **a** | all tissues; **b** | sectors of tissue; or **c** | the cotyledons, but not the rest of the plant. Images courtesy of C.A.H. and P.M.W., CSIRO, Australia. Reproduced with permission from REF. 63 © (2002) CSIRO Publishing.

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cloning, the transfer of gene inserts into Gateway vectors is reliable and efficient, enabling numerous inserts to be moved to the vector of choice (BOX 1). Gateway-cloned *Arabidopsis* cDNA libraries (see link to Construction of an *Arabidopsis* open reading frame library) and *Arabidopsis* gene sequence tags (BOX 1) are obvious sources of the gene fragments that are required for RNAi-based studies of gene function. Coordinated genomic investigations built around these libraries can be envisaged. Experiments using microarrays are beginning to identify many of the genes that show expression changes in response to external stimuli[67], that are expressed in a particular tissue[68] or that are altered in a mutant[69]. So far, RNAi in nematodes has revealed phenotypes and possible functions for 13–27% of the ~7,000 genes examined, including gene products with previously unknown biochemical properties[64–66]. Hopefully, similar insights will be made into the functions of plant genes.

Although Gateway cloning and the pHELLSGATE vectors can facilitate the rapid and automatable generation of RNAi constructs (BOX 1), the stable delivery of these transgene libraries into plants is only practically feasible in *Arabidopsis*, for which an easy, non-tissue-culture, floral-dip method of transformation is available. Fortunately, *Arabidopsis* is now the foremost plant for genomic research, given the wealth of genomic information and resources that are available for it (see the link to TAIR). Before hpRNA transgenes can be used for genome-wide research in any other plant species, similarly easy and efficient transformation systems will need to be developed.

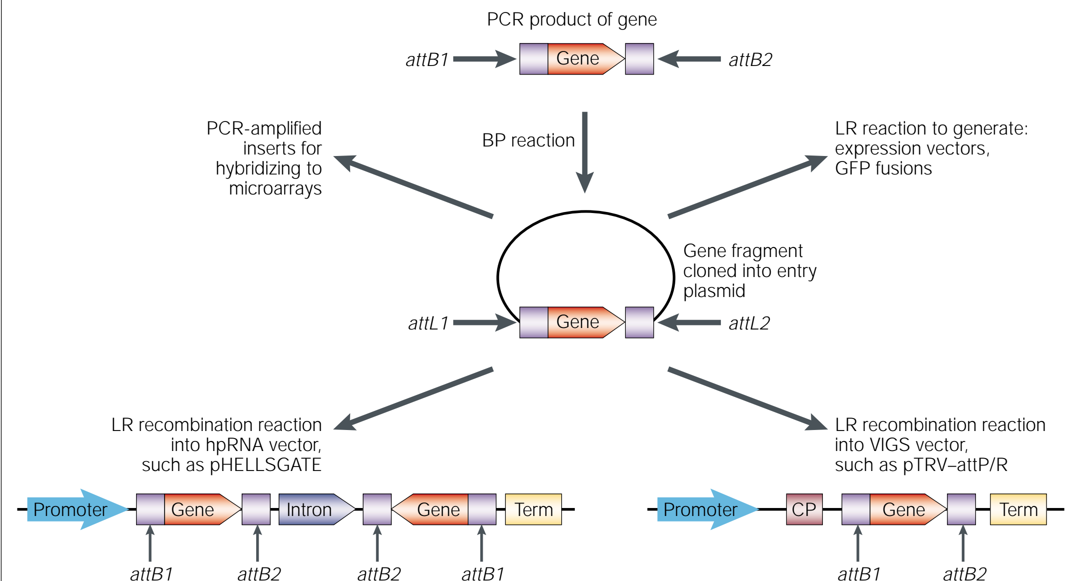
Box 1 | Applications of RNA interference in plant genomics

RNA interference (RNAi) is beginning to be used as a tool for plant genomic research, particularly in *Arabidopsis*, which is now the plant species with the most extensive array of genomic resources.

Several projects are under way to produce the resources that are required for RNAi to be used as a tool for high-throughput plant genomics research. The CATMA group (Complete *Arabidopsis* Transcriptome MicroArray; see the link to CATMA) is generating a set of PCR products, called gene sequence tags (GSTs), that represent each *Arabidopsis* gene and have been designed to hybridize in a gene-specific manner on *Arabidopsis* cDNA microarrays. The primers that are used to generate these PCR products have 5′ extensions that enable them to be reamplified and cloned into Gateway vectors by a BP clonase reaction (see figure). The AGRIKOLA consortium (*Arabidopsis* genomic RNAi knock-out line analysis; see link for more information on this project) is using this set of PCR products to generate, in pHELLSGATE vectors through an LR clonase reaction, a gene-specific RNAi construct for each *Arabidopsis* gene to be used in large-scale RNAi-silencing studies — a resource that will complement whole-genome microarrays. The gene fragments that have been cloned into these entry vectors could also be recombined into VIGS (viral-induced gene silencing) vectors, such as pTRV–attR (REF. 44), by an LR clonase reaction (see figure).

Identifying gene function by RNAi-induced gene silencing in plants is a key challenge for the future because many gene knockouts in plants do not produce a phenotype under normal growth conditions. Sets of growth conditions (the so-called 'Gauntlet'), in which, for example, temperature, nutrients, light quality and hormones are varied, are being developed for *Arabidopsis* (see link to the *Arabidopsis* Gauntlet Project). The aim of this type of screen is to reveal mutant phenotypes that are specific to particular growth conditions — the first step in assigning a function to a gene. The highly automated approach to identifying agriculturally useful genes that is being taken by CropDesign (see online link for more on this approach) also highlights the type of analysis that will be needed to identify such phenotypes on a large scale.

***attB1, attB2, attL1, attL2*, Gateway recombination sites; CP, coat protein; GFP, green fluorescent protein; Term; transcription termination sequence.**



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Agro-VIGS is free from the limitations imposed by plant transformation efficiency, and the pTRV–attP/R vectors enable the rapid generation of gene silencing libraries (BOX 1). TRV–VIGS has been most widely used in *N. benthamiana*, for which transposons or chemically induced mutants are not widely available and about which little classical genetic or sequence information exists. However, TRV–Agro-VIGS seems to be equally effective in the tomato[44], for which there is a good genetic map, a wide range of spontaneous and induced mutants and large BAC (bacterial artificial chromosome) and EST (expressed sequence tag) libraries. For species that are difficult to transform, VIGS is the system of choice. Cereal species in particular fit this category; progress in this area depends on finding suitable virus vectors. However the demonstration of VIGS in barley using barley stripe mosaic virus (BSMV)[38] indicates that similar vectors should ultimately be found for all cereals.

It is likely that genome-scale RNAi studies using hpRNA constructs will initially be carried out using strong constitutive promoters to give a rapid survey of gene function. However, hpRNAs also offer the possibility of directing silencing in specific tissues depending on the promoter used to direct expression of the hpRNA. This type of approach would be particularly useful in defining the role of genes that give pleiotropic phenotypes when function is impaired at the whole plant level. Similarly, chemically induced promoter systems[70] could also be used to direct expression of hpRNAs. This type of system would be useful for defining the functions of genes that give embryonic lethal phenotypes when mutated and also for studying the roles of genes at specific stages in plant development. RNAi approaches also offer the prospect of silencing whole gene families or several, unrelated genes by either targeting conserved regions of nucleic acid sequence or including several target sequences in the same RNAi-inducing construct, respectively.

We hope that we have shown that RNA silencing has many features that make it a system of choice for plant functional genomics. The flexibility it offers is likely to be important in understanding many aspects of the function of the *Arabidopsis* genome. As plant research moves on to the challenges of understanding the biology of crop plants such as wheat — in which genome size, complexity and redundancy obviate the use of single-gene mutagenesis — RNA silencing, in some form, will probably have an even greater role.

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