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Invited Review

RNA silencing manifested as visibly altered phenotypes in plants

Akira Kanazawa*

Research Faculty of Agriculture, Hokkaido University, Sapporo 060-8589, Japan
* E-mail: kanazawa@res.agr.hokudai.ac.jp Tel: +81-11-706-3873 Fax: +81-11-706-4933

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Abstract Eukaryotes have a mechanism of RNA-guided regulation of gene expression in which double-stranded RNA inhibits the expression of genes with complementary nucleotide sequences. This mechanism plays a crucial role in many processes including development, stability of the genome, and responses against invading genetic materials. These RNA-guided pathways that control gene expression, collectively termed RNA silencing, are thought to have evolved as a form of innate immunity against viruses. RNA silencing provides a mechanism for downregulating gene expression and a tool that is suitable for analyzing gene function and engineering novel traits in organisms. The phenomenon of RNA silencing was discovered in transgenic petunia plants that had altered patterns of flower color as a consequence of overexpression of the *chalcone synthase-A* gene responsible for an essential enzyme to biosynthesize anthocyanins. After the "visual" discovery of RNA silencing in petunia, visible phenotypes have played an important role in monitoring the silenced state of a gene in various RNA silencing systems. In particular, a photobleached phenotype in leaf tissues is useful in optimizing a virus-induced gene silencing system. Loss of pigmentation in plant tissues has also led to the detection of naturally occurring RNA-silencing phenomena. Visual changes conferred by endogenous reporter genes provide highly informative assessments of RNA silencing that can be applied to a wide spectrum of plant biotechnology.

Key words: Endogenous reporter gene, RNA interference, RNA silencing, virus-induced gene silencing.

Biologists often use a reporter gene to monitor gene expression and/or subsequent behavior of proteins in cells. The availability of reporter genes, in combination with efficient transformation methods, makes biological processes with subtle or hidden phenotypes accessible to forward genetic approaches. For example, the identification of important genes involved in the circadian system, hormone signaling pathways, and plant responses to biotic and abiotic stresses have been achieved by screening mutants with altered expression of a reporter gene (reviewed by Page and Grossniklaus 2002). This elucidative potency of reporter genes is also true in the studies of RNA silencing, in which genes necessary for induction of RNA silencing have been isolated by screening mutants that break the silent state of the reporter transgene (Matzke et al. 2001; Page and Grossniklaus 2002).

The discovery of RNA silencing was preceded by reports of unexpected outcomes in experiments by plant scientists in 1990 (Napoli et al. 1990; Van der Krol et al. 1990). In this case, the "reporters" were endogenous genes. In an attempt to test whether the encoded enzyme is rate limiting in anthocyanin biosynthesis, that is manifested as particular flower colors, additional copies of a gene encoding chalcone synthase (CHS) or dihydroflavonol-4-reductase (DFR) were introduced into petunia (*Petunia hybrida*) plants. The overexpressed gene was expected to result in darker purple flowers, but instead produced flowers with white sectors in purple background or completely white flowers, indicating that the activity of chalcone synthase had substantially decreased (Figure 1A). Analysis of RNA indicated that both the endogenous genes and the transgenes were downregulated in the white sectors. The term co-suppression was coined to refer to the phenomenon (Napoli et al. 1990).

Similar phenomena were detected in plants transformed with various genes. These include tomato plants transformed with a construct transcribing a truncated polygalacturonase gene (Smith et al. 1990) and tobacco plants transformed with the gene for β-1,3-glucanase (de Carvalho et al. 1992), nopaline synthase (Goring et al. 1991), or nitrate reductase (Vaucheret 1993). A related phenomenon termed quelling was also reported in the fungus *Neurospora crassa*: an introduction of extra copies of genes essential for biosynthesis of a carotenoid pigment resulted in an unpigmented mould rather than a more intense orange one (Cogoni and Macino 1997). The inhibition of gene activity by the introduction of a transgene into plants indicated that the inhibition took place at the transcriptional level (transcriptional gene silencing; TGS) (Matzke et al. 1989; Wassenegger et al.

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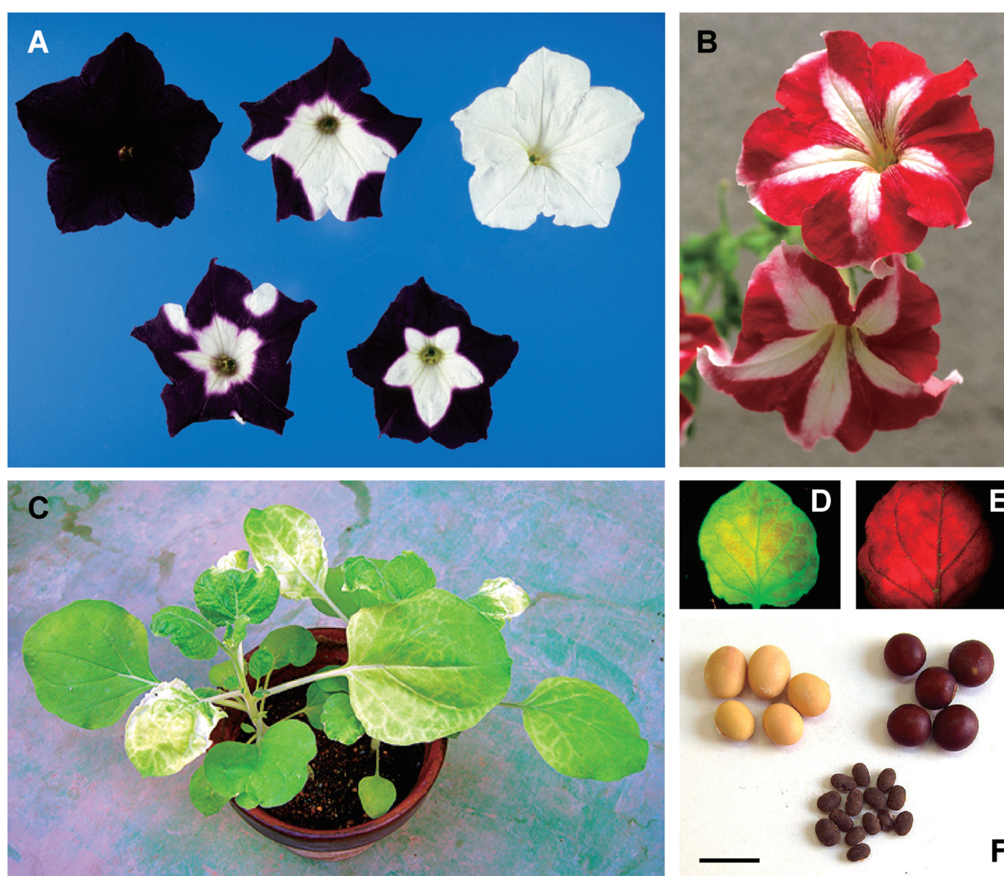

Figure 1. Examples of visible phenotypic changes as a consequence of RNA silencing in plants. (A) Various flower color patterns of transgenic petunia as a consequence of *CHS-A* co-suppression. Nontransgenic plants of line V26 produce totally purple flowers (upper left), whereas overexpression of the *CHS-A* gene causes the production of white sectors or completely white flowers in transformed plants (the others). The transgene was integrated in the genome as an inverted repeat in the plant line with the totally white flowers (upper right), which probably accounts for the stable RNA silencing in this line (Kanazawa et al. 2007). (B) Flowers of petunia 'Red Star' with a star-type, red and white bicolor pattern. The formation of white sectors in this nontransgenic plant is caused by RNA silencing of the *CHS-A* gene (Koseki et al. 2005). (C) *N. benthamiana* plant with photobleached phenotype as a consequence of *PDS* VIGS. (D, E) Changes in GFP fluorescence by VIGS in leaf tissues of *N. benthamiana*. Fluorescence of GFP produced by the expression of the transgene integrated in the genome is unchanged by infection with virus containing no insert (D), but was lost by infection with virus containing a portion of the *GFP*-coding region (E) (Otagaki et al. 2006). The red color in the *GFP*-silenced tissue comes from autofluorescence of chloroplasts, which is masked by GFP fluorescence when the *GFP* transgene is expressed. Similar results were obtained when plants were infected with virus carrying a portion of the transgene-promoter (Otagaki et al. 2006). (F) Seeds of cultivated soybean and wild soybean. Cultivated soybean consists of varieties that produce yellow (upper left), brown (upper right) or black seed coats, whereas the wild ancestor of cultivated soybean exclusively produces pigmented seed coats (lower). The yellow seed coat color of cultivated soybean is caused by RNA silencing of the *CHS* genes (Senda et al. 2004). A bar indicates 10 mm.

1994; Park et al. 1996), or at the posttranscriptional level (posttranscriptional gene silencing; PTGS) (Napoli et al. 1990; van der Krol et al. 1990; Smith et al. 1990; de Carvalho et al. 1992; van Blockland et al. 1994). Based on the observation of the systemic spread of the loss of fluorescence of green fluorescent protein (GFP) after induction of *GFP* PTGS by *Agrobacterium* infiltration (Voinnet and Baulcombe 1997) or the acquisition of the chlorosis phenotype induced by nitrate reductase PTGS through grafting (Palauqui et al. 1997), the occurrence of systemic signaling of PTGS was assumed.

A similar unexpected phenomenon was observed in studies of plant resistance to viral diseases. While plants that expressed genes derived from a virus were known to have enhanced resistance to viral infection (reviewed by Wilson 1993; Baulcombe 1996a), plants carrying noncoding viral RNA sequences also had similar levels of protection (Smith et al. 1994; Mueller et al. 1995; Sijen et al. 1996). Viral RNA produced by transgenes was believed to inhibit viral replication via gene silencing.

Prompted by the findings in plants, workers searched for gene silencing phenomenon analogous to co-suppression and actually detected it in *Drosophila* (Pal-Bhadra et al. 1997). RNA–RNA pairing was considered to be crucial for inducing RNA degradation based on the studies of co-suppression in petunia (Metzlaff et al. 1997). A potent gene silencing effect (e.g., twitching movement phenotype) was detected when double-stranded RNA (dsRNA) was injected into cells in

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Caenorhabditis elegans (Fire et al. 1998), and the phenomenon was called RNA interference (RNAi). An unexpected gene silencing phenomenon induced by sense RNA, instead of antisense RNA, had also been reported in *C. elegans* (Guo and Kemphues 1995). The inhibitory effect of sense RNA in this report was presumed to be due to the joint effect of the sense RNA with antisense RNA that may have co-existed in the sense RNA preparation (Fire et al. 1998). It was also shown in plants that dsRNA initiates PTGS (Waterhouse et al. 1998) and that PTGS is correlated with the production of a population of small RNAs that contain both sense and antisense RNA (Hamilton and Baulcombe 1999).

To understand the mechanisms of gene silencing, researchers then isolated genes responsible for the phenomena by screening mutants deficient in the induction of gene silencing in *N. crassa*, *C. elegans*, and *Arabidopsis* (reviewed by Matzke et al. 2001). In combination with biochemical experiments using cellular extracts of *Drosophila*, the degradation of target mRNA was found to comprise primarily a two-step process: (1) RNaseIII-type dsRNA endonuclease activity that processes dsRNA into short interfering RNA (siRNA) of 21 to 26 nucleotides long, and (2) cleavage ("slicing") of target RNA by RNA-induced silencing complexes (RISCs) that contain a member of the Argonaute (Ago) proteins. Ago proteins have an RNA-binding PAZ domain and an endonucleolytic PIWI domain. In this complex, the antisense siRNAs serve as guides for the cleavage site (Matzke et al. 2001) (Figure 2).

In addition to the silencing of a specific gene via the slicing and subsequent degradation of mRNA, the production of siRNAs was also found to be associated with induction of epigenetic changes in nuclei involving cytosine methylation and changes in the state of histone modifications (reviewed by Matzke et al. 2004). Furthermore, like siRNAs, small RNAs called micro RNAs (miRNAs) were found to negatively regulate the expression of endogenous genes through either RNA cleavage or the arrest of translation, which is another pathway of RNA silencing (reviewed by Baulcombe 2004; Mallory and Vaucheret 2006). Based on these findings, gene silencing phenomena that are induced by nucleotide sequence-specific interactions mediated by RNA are collectively called RNA silencing (Voinnet 2002; Matzke et al. 2004). The highly diverse pathways of RNA silencing so far known have been reviewed in detail elsewhere (Brodersen and Voinnet 2006; Vaucheret 2006).

Transgene-induced RNA silencing as a tool for engineering metabolic pathways

The understanding of the general mechanisms of the RNAi process has prompted plant scientists to use RNAi technology to modify various traits in plants. Although the phenomenon of co-suppression was discovered in petunia plants in which a transgene expressing sense RNA was introduced, it became evident that efficient production of dsRNA for a target gene is associated with the efficient induction of RNAi. In fact, transgenes are often integrated into the genome as an inverted repeat structure, which may produce dsRNA when read-through transcription occurs, in plants with co-suppression (Cluster et al. 1996; Stam et al. 1998; Muskens et al. 2000).

While we can transform plants independently with constructs that produce sense and antisense RNA and then cross these sense and antisense plants to get progeny that express both these RNAs and thus induce RNAi (Waterhouse et al. 1998), a widely used method to produce dsRNA in plant cells is to transform plants with a construct comprising an inverted repeat (IR) sequence of the target gene, which forms dsRNA upon transcription (IR-PTGS; Smith et al. 2000; Wesley et al. 2001; Helliwell and Waterhouse 2005). RNA silencing induced by a transgene transcribing IR sequence involves fewer factors than that by a transgene transcribing sense RNA (Béclin et al. 2002) (Figure 2).

At present no definitive constraint for application of RNAi technology to downregulate expression of a gene has been reported as long as the source of dsRNA has sufficient length and sequence identity with the target RNA; hence, the method can potentially be used to downregulate any genes. Various genes in metabolic pathways in plants have been a target for engineering by this approach. Such alterations include a reduction in the content of specific compounds. In addition, accumulation of useful compounds by targeting genes involved in the downstream or branched steps of the pathway has also been achieved. Various transformed plants have been produced by utilizing co-suppression or IR-PTGS (reviewed by Flavell 1994; Baulcombe 1996b; Mansoor et al. 2006; Tang and Galili 2004). Recent examples of plant engineering by IR-PTGS include a reduction in caffeine by targeting genes involved in caffeine biosynthetic pathway in coffee bean plant (Ogita et al. 2004), the accumulation of non-narcotic alkaloid by targeting the codeine reductase gene in opium poppy (Allen et al. 2008), the accumulation of reticuline by targeting a berberine bridge enzyme gene in California poppy (Fujii et al. 2007), the accumulation of lysine by targeting a lysine degrading enzyme gene in maize (Houmard et al. 2007), the production of gossypol toxin-free oil by targeting a gene involved in its biosynthesis in cotton (Sunilkumar et al. 2006), and the induction of male sterility by targeting anther-specific genes in rice (Moritoh et al. 2005).

TGS can also be induced by producing dsRNA

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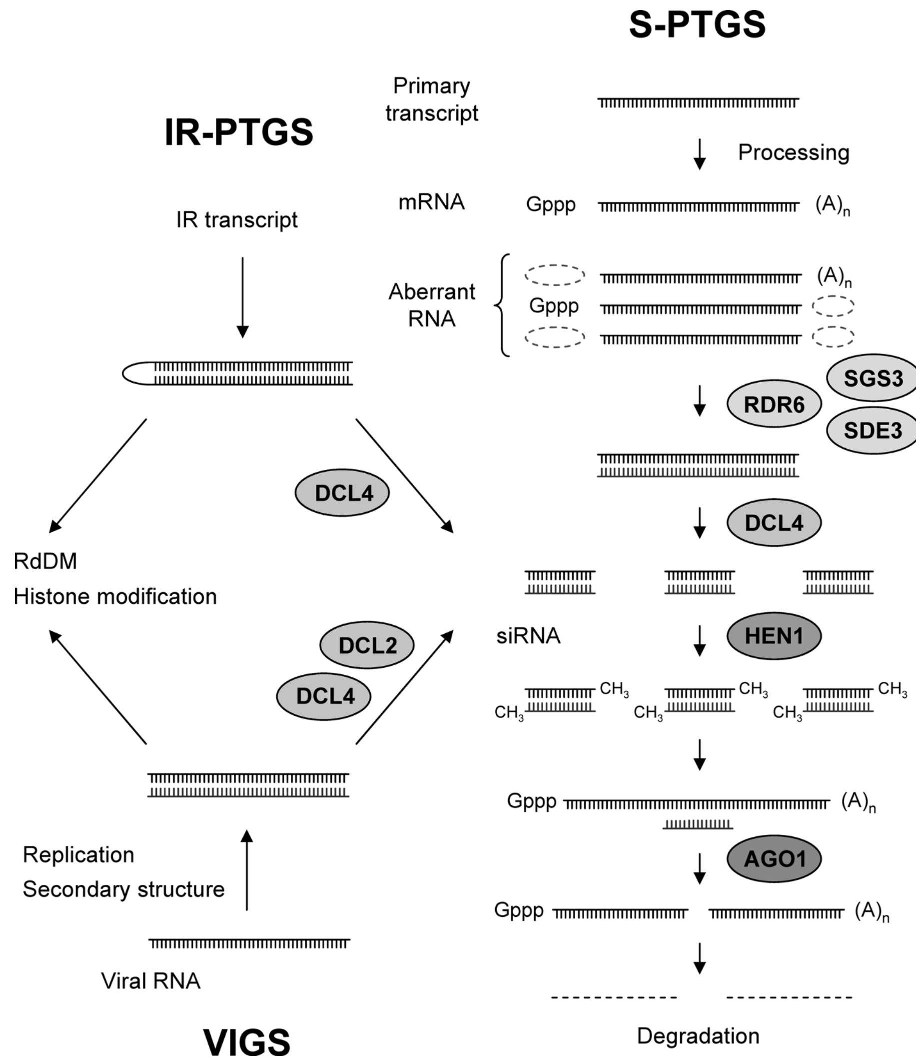

Figure 2. Pathways of RNA silencing used in the engineering of novel traits in plants. Posttranscriptional gene silencing triggered by sense RNA (S-PTGS; from upper right) involves the production of dsRNA from aberrant RNA lacking 5′ capping and/or polyA tail structures via the function of RNA-dependent RNA polymerase RDR6 (Dalmay et al. 2000; Mourrain et al. 2000). SGS3, a protein with a coiled-coil motif and an RNA-stabilizing function (Mourrain et al. 2003), and SDE3, a DEAD-box RNA helicase (Dalmay et al. 2001), are also involved in this process. The dsRNA is processed by a Dicer-like (DCL) protein, possibly DCL4 (Brodersen and Voinnet 2006), producing siRNAs of 21 nt in size, which is then methylated by HUA ENHANCER1 (HEN1; Boutet et al. 2003), a plant specific methyltransferase. The siRNAs are involved in the cleavage of target mRNA with RISCs that contain AGO1 (Fagard et al. 2000; Morel et al. 2002), which subsequently leads to the degradation of the cleaved mRNA. The cleaved mRNA can be further used as a source of dsRNA production. When PTGS is triggered by a transgene transcribing an inverted repeat sequence that can form dsRNA (IR-PTGS; from upper left toward right), RDR6, SGS3, and SDE3 are not required (Béclin et al. 2002), and the siRNAs are produced from the dsRNA by DCL4 (Dunoyer et al. 2005). Both S-PTGS and IR-PTGS may account for co-suppression; IR-PTGS can be induced by read-through transcription over duplicated copies of a transgene if the transgene is integrated in the genome as a form of an inverted repeat. Viral RNA is known to be processed by DCL2 and DCL4, in which the processing activity of DCL2 is subordinate to that of DCL4 (Deleris et al. 2006; from lower left to right). The siRNAs in the VIGS pathway are probably produced by these enzymes and used for the endonucleolytic cleavage of mRNA with RISCs. VIGS is abolished in the *dcl2-dcl4* double mutant (Deleris et al. 2006). The following pathways, which are assigned to different DCL proteins, are also known. DCL1, together with dsRNA-binding protein HYL1, processes fold-back precursors to release miRNAs in nucleus, which are exported to cytoplasm by HASTY (HST) and subsequently lead to endonucleolytic cleavage of homologous mRNA with AGO1-loaded RISCs or inhibition of translation (reviewed by Mallory and Vaucheret 2006; not shown in this figure). DCL3 produces 24-nt siRNAs that guide RdDM and changes in the state of histone modification, which involves the functions of RDR2, AGO4, Pol IVa, Pol IVb, DNA methyltransferases (MET1, CMT3, and DRM1/2), histone deacetylase HDA6, histone methyltransferase KYP (SUVH4), and chromatin-remodeling factor DRD1 (reviewed by Matzke et al. 2004; Matzke and Birchler 2005; from upper or lower left to left). DCL4 is also involved in the processing of dsRNA produced by RDR6 subsequent to the endonucleolytic cleavage of precursor RNAs with miRNAs, which results in the production of a species of siRNA called transacting siRNAs (ta-siRNAs; Peragine et al. 2004; Vazquez et al. 2004; not shown in this figure) that guide cleavage of homologous mRNA, once incorporated into AGO1-loaded RISCs. All known classes of short RNA in plants are methylated by HEN1 (right). All protein names in this figure are those in *Arabidopsis*, identified by screening mutants deficient in the pathways of RNA silencing.

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corresponding to a promoter sequence via an induction of DNA methylation, a phenomenon called RNA-directed DNA methylation (RdDM) and/or via changes in the state of histone modification. Using a transgene that transcribes the IR sequence of a promoter, scientists have induced TGS of transgenes (Mette et al. 1999, 2000) or endogenous genes such as the petunia *DFR* gene (Sijen et al. 2001), the maize male fertility gene *Ms45* (Cigan et al. 2005), the potate granule-bound starch synthase I gene (Heilersig et al. 2006), and the rice *Se5* gene (Okano et al. 2008).

In addition, a recently introduced approach in plants is the use of artificial miRNAs (amiRNAs; also called synthetic miRNAs; reviewed by Ossowski et al. 2008). This approach involves modification of plant miRNA sequence to target specific transcripts, originally not under miRNA control, and downregulation of gene expression via specific cleavage of the target RNA. This method has been applied to target viral RNA (Niu et al. 2006) and transcripts of endogenous genes in plants (Schwab et al. 2006; Alvarez et al. 2006).

Virus-induced gene silencing: utilization of RNA-mediated defense mechanism against virus

Several lines of research indicated that RNA silencing is a general antiviral mechanism in plants. The effects of gene silencing in plants were first used to develop resistance to viral diseases, even though the mechanism was not clear at the time. Resistance to virus was achieved by transforming plants with genes or segments of genes derived from viruses and was referred to as pathogen-derived resistance (reviewed by Wilson 1993; Baulcombe 1996a; Prins and Goldbach 1996; Goldbach et al. 2003). Transgenic tobacco plants expressing genes from *Tobacco etch virus* (Linbdo et al. 1993; Dougherty et al. 1994), *Potato virus Y* (Smith et al. 1994), *Potato virus X* (Mueller et al. 1995), or *Cowpea mosaic virus* (Sijen et al. 1996) acquired immunity, and the resistance did not need protein translated from the transgene (Smith et al. 1994; Mueller et al. 1995; Sijen et al. 1996), which led to the understanding that RNA is the factor that conferred resistance to the plants. Use of transgene-induced RNAi technology for plants to acquire resistance against virus has been reported for various combinations of plants and viruses (reviewed by Baulcombe 1996a; Mansoor et al. 2006; Goldbach et al. 2003).

Although transgenes expressing a virus-derived gene or gene segment confer enhanced resistance against virus via a mechanism analogous to that involved in co-suppression, plants intrinsically have the ability to cope with viruses. When plants are infected with an RNA virus, dsRNA of the viral genome is degraded by the infected plants (Covey et al. 1997; Al-Kaff et al. 1998). The dsRNA in the virus-infected cells is thought to be the replication intermediate of the viral RNA (Lu et al. 2003). A recent report suggests that the single-stranded RNA of the viral genome forms the secondary structure (Molnar et al. 2005). The viral genomic RNA can be processed into siRNAs, then targeted by the siRNA/RNase complex. In this scenario, if a nonviral segment is inserted in the viral genome, siRNAs would also be produced from the segment. Therefore, if the insert corresponds to a sequence of the gene encoded in the host plant, infection by the virus results in the production of siRNAs corresponding to the plant gene and subsequently induces loss of function of the gene product. This fact led to the use of virus vector as a source to induce silencing of a specific gene in the plant genome, which is referred to as virus-induced gene silencing (VIGS; Kumagai et al. 1995; Ruiz et al. 1998).

The role of outward phenotypes in establishing a VIGS system

To monitor successful induction of VIGS, a reporter transgene expressed stably in plants has often been used (Figure 1D, 1E). With regard to the VIGS of an endogenous gene encoded by the plant genome, the gene for phytoene desaturase (PDS) is often chosen as a target because loss of function of this gene is manifested as a photobleached phenotype (Figure 1C). VIGS of these "visible" genes revealed that the extent of the induction of silencing is not equivalent between different portions of virus-infected plants because induction of the silencing is associated with propagation of virus in the host plants. This conditional nature of VIGS may have both positive and negative aspects in terms of using the technology for functional genomics. The instability may be a negative aspect of VIGS. However, this in turn may be an advantage by allowing observation of phenotypic changes caused by dysfunction of a gene whose complete loss of expression is lethal to the plant (Lu et al. 2003). In fact, phenotypic changes have been induced by VIGS of the gene for proliferating cell nuclear antigen (Peele et al. 2001) and RNA polymerase II (Gosselé et al. 2002), for which null mutants cannot be retrieved by conventional or insertional mutagenesis approaches.

When VIGS is used to analyze the function of a gene, viral infection itself might be a problem depending on the target gene. Symptoms of virus infection indicate that gene expression in the infected cells has been affected. If a gene with expression affected by viral infection is chosen as the target of VIGS, the effect of VIGS might not appear as a specific effect caused by the sequence-specific degradation of the RNA, but a nonspecific effect of the viral infection might also be involved. Accordingly, efforts are sometimes needed to

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reduce the extent of nonspecific effects of viral infection and simultaneously establish efficient induction of VIGS. In this respect, *Nicotiana benthamiana* is the plant species most frequently used for VIGS experiment. This plant, sometimes referred to as a model plant for plant virus study, is suitable for induction of VIGS because of the large exclusion limit through its plasmodesmata (Lu et al. 2003). It has also been suggested that this plant lacks an active salicylic acid- and virus-inducible RNA-dependent RNA polymerase (RdRP) and thus is hypersusceptible to viruses whose accumulation is normally limited by this RdRP (Yang et al. 2004). In contrast, when a new combination of plant species and virus vector is used, it is often necessary to control the efficiency of viral infection and symptom production to optimize the induction of VIGS. On such occasions, genes such as *PDS* whose silencing is manifested as an altered visible phenotype are chosen as the target of VIGS (Table 1). Once the system is established in the plant, the method can be applied to various genes. Applications of virus-induced gene silencing have been reviewed elsewhere (Lu et al. 2003; Burch-Smith et al. 2004). A comprehensive list of VIGS vectors and the plants and genes to which VIGS have been applied so far is given in Table 1. VIGS that accompanies neither severe viral symptoms nor phenotypic changes has been achieved, for example, by targeting the flavonoid 3′-hydroxylase (*F3′H*) gene in soybean, in which symptomless infection of virus is established by the use of a pseudorecombinant virus, and the flavonoid content was successfully modified by VIGS (Nagamatsu et al. 2007).

It should also be noted that a virus vector carrying a promoter sequence can induce transcriptional silencing of a target gene through RdDM of the promoter of transgene integrated in the plant genome. This case has been achieved with *Potato virus X* (Jones et al. 1999), *Tobacco rattle virus* (Jones et al. 2001) and *Cucumber mosaic virus* (Otagaki et al. 2006) by targeting the CaMV 35S promoter integrated in the genome upstream of the *GFP* gene that allows detection of changes in promoter activity. In contrast to VIGS causing mRNA degradation, the silenced state of the transgene induced by the transcriptional VIGS can be heritable in the progeny to which no virus is transmitted. However, no plant has so far been produced that harbors a silenced endogenous gene by promoter-targeting VIGS, which reflects the difficulty in inducing epigenetic changes in an endogenous gene and restricts the practical application of this method.

Use of viral suppressor protein in the study of RNA silencing

Another interesting aspect of the use of viruses for the study of gene silencing in plants is the function of a virus-encoded suppressor protein of RNA silencing. These suppressor proteins affect viral accumulation in plants: whether a virus accumulates at a high level depends on the ability of the suppressor protein. The ability of the suppressor protein to allow viral accumulation is due to its inhibition of RNA silencing by preventing the incorporation of siRNAs into RISCs or by interfering with RISCs (reviewed by Silhavy and Burgyan 2004). Using the function of viral suppressor protein, we can "diagnose" whether an observed phenotypic change in a plant is caused by RNA silencing (described later). Viral suppressor protein can also be used to obtain a high level of gene expression in a transient expression system by preventing the onset of PTGS (Voinnet et al. 2003).

Naturally occurring RNA silencing and its phenotypes

Artificially induced RNA silencing is not the only biological phenomenon for which visual phenotypes had played an important role in increasing our understanding. Naturally occurring RNA silencing, involving mRNA degradation induced as a consequence of certain genetic changes, has been detected based on phenotypic changes. Most commercial varieties of soybean produce yellow seeds due to loss of pigmentation in seed coats, and this phenotype has been shown to be due to PTGS of the *CHS* genes (Senda et al. 2004). In cultivated soybean (*Glycine max*), there are varieties producing seeds with yellow seed coats and those producing seeds with brown or black seed coats in which anthocyanin and proanthocyanidin accumulate (Figure 1F). In contrast, wild soybeans (*Glycine soja*), an ancestor of the cultivated soybean, have exclusively produced seeds with pigmented seed coats (Figure 1F) in thousands of accessions from natural populations in East Asia that we have screened (unpublished data). Thus, the nonpigmented seed coat phenotype was probably generated after domestication of soybean, and humans have maintained the plant lines that have *CHS* RNA silencing. The genetic change that induced *CHS* RNA silencing has been attributed to a structural change in the *CHS* gene cluster, which allows production of inverted repeat *CHS* RNA (Kasai et al. 2007). A similar association between structural changes in DNA and the occurrence of RNA silencing that leads to changes in pigmentation of plant tissues has also been reported for the *CHS* genes in maize (Della Vedova et al. 2005).

Petunia 'Red Star' is a variety with flowers having a star-type red and white bicolor pattern (Figure 1B). The star-type pattern resembles the flower-color patterns observed in transgenic petunias with co-suppression of the *CHS* genes (Jorgensen 1995), and in fact, the

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Table 1. VIGS vectors

Virus	Plant species	Target gene	Reference
RNA virus			
TMV	*N. benthamiana*	*PDS*	Kumagai et al. 1995
		PDS, *GFP*	Lacomme et al. 2003
PVX	*N. benthamiana*	*PDS*, *GFP*	Ruiz et al. 1998
		rbcS, *GFP*	Jones et al. 1999
		CesA	Burton et al. 2000
		CDPK	Romeis et al. 2001
		FtsH	Saitoh and Terauchi 2002
		WIPK, *SIPK*	Sharma et al. 2003
		Randomly chosen 4992 genes	Lu et al. 2003
		RdRP	Yang et al. 2004
	Potato	*PDS*	Faivre-Rampant et al. 2004
TRV	*N. benthamiana*	*GFP*, *GUS*, *Leafy*	Ratcliff et al. 2001
		PDS, pathogen responsive genes	Liu et al. 2002b
	Tomato	*PDS*, *rbcS*, ethylene responsive genes, etc	Liu et al. 2002a
	Pepper	*PDS*, *rbcS*	Chung et al. 2004
	Petunia	*PDS*, *CHS*, *ACO*	Chen et al. 2004
	Four species in the genus *Solanum*	*PDS*, disease resistance genes	Brigneti et al. 2004
	N. benthamiana	*PDS*, 20S proteasome subunit gene, *Chl H*	Ryu et al. 2004
	Tomato		
	Pepper		
	Tobacco		
	Potato		
	Petunia		
	N. benthamiana	Genes expressed in roots	Valentine et al. 2004
	Arabidopsis		
	Tomato		
	Tomato	Ethylene responsive genes, fruit ripening genes	Fu et al. 2005
	Opium poppy	*PDS*	Hileman et al. 2005
	Arabidopsis	*PDS*, *CH42*, *GFP*, *CUL1*, disease resistance genes	Burch-Smith et al. 2006
	Arabidopsis	*PDS*, disease resistance genes	Cai et al. 2006
	N. benthamiana	Stress responsive genes	Senthil-Kumar et al. 2007a
STMV	Tobacco	*PDS*, *CHS*, *rbcS*, *tk*, *CesA*, *als*, *pol II*, *cat*, etc	Gosselé et al. 2002
TBSV	*N. benthamiana*	*GFP*	Qiu et al. 2002
BSMV	Barley	*PDS*	Holzberg et al. 2002
		PDS	Lacomme et al. 2003
		PDS, disease resistance genes	Hein et al. 2005
	Wheat	*PDS*, disease resistance genes	Scofield et al. 2005
PEBV	Pea	*PDS*, *UNIFOLIATA*, *KORRIGAN1*	Constantin et al. 2004
ToMV	*N. benthamiana*	*PDS*	Hori et al. 2004
		PMT	Takizawa et al. 2007
BPMV	Soybean	*PDS*	Zhang and Ghabrial 2006
CMV	*N. benthamiana*	*GFP*	Otagaki et al. 2006
	Soybean	*CHS*, *F3′H*	Nagamatsu et al. 2007
BMV	Barley	*PDS*, *actin*	Ding et al. 2006
	Rice		
	Wheat		
DNA virus			
TYDV	Petunia	*CHS*	Atkinson et al. 1998
TGMV	*N. benthamiana*	*su*, *LUC*	Kjemtrup et al. 1998
		su, *GFP*, *PCNA*	Peele et al. 2001
CbLCV	*Arabidopsis*	*PDS*, *CH42*	Turnage et al. 2002
ACMV	Cassava	*PDS*, *su*, *CYP79*	Fofana et al. 2004
TYLCCNV	*N. benthamiana*	*PDS*, *su*, *PCNA*, *GFP*	Tao and Zhou 2004

Abbreviations of virus names: TMV, *Tobacco mosaic virus*; PVX, *Potato virus* X; TRV, *Tobacco rattle virus*; STMV, *Satellite tobacco mosaic virus*; TBSV, *Tomato bushy stunt virus*; BSMV, *Barley stripe mosaic virus*; PEBV, *Pea early browning virus*; ToMV, *Tomato mosaic virus*; BPMV, *Bean pod mottle virus*; CMV, *Cucumber mosaic virus*; BMV, *Brome mosaic virus*; TYDV, *Tobacco yellow dwarf virus*; TGMV, *Tomato golden mosaic virus*; CbLCV, *Cabbage leaf curl virus*; ACMV, *African cassava mosaic virus*; TYLCCNV, *Tomato yellow leaf curl China virus*.

Abbreviations of gene names: PDS, phytoene desaturase; GFP, green fluorescent protein; rbcS, small subunit of ribulose-bisphosphate carboxylase/oxygenase; CesA, cellulose synthase A; CDPK, calcium-dependent protein kinase; FtsH, FtsH protease; WIPK, wound-induced protein kinase; SIPK, salicylic acid-induced protein kinase; RdRP, RNA-dependent RNA polymerase; GUS, β-glucuronidase; *CHS*, chalcone synthase; ACO, 1-aminocyclopropane-1-carboxylate oxidase; Chl H, Mg-protoporphyrin chelatase; CH42, Chlorata 42; CUL1, Cullin 1; tk, plastid transketolase; als, acetolactate synthase; pol II, RNA polymerase II; cat, catalase; PMT, putrescine N-methyltransferase; F3′H, flavonoid 3′-hydroxylase; su, sulfur; LUC, luciferase; PCNA, proliferating cell nuclear antigen; CYP79, cytochrome P-450 CYP79.

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phenotype was demonstrated to be due to RNA silencing of the *CHS* genes in the white sectors (Koseki et al. 2005). Breeding of petunia was launched in the 1830s by crossing among wild species. The generation of the star-type petunia plants as a consequence of hybridizations between plant lines suggests that RNA silencing ability can be conferred via shuffling of genomes that are slightly different from each other.

The silencing phenomena in soybean, maize, and petunia all involve RNA silencing of the *CHS* genes and consequently changes in pigmentation of plant tissues. In the analyses of these processes, the function of the viral suppressor protein was used to visually demonstrate the occurrence of RNA silencing of plant endogenous genes (Senda et al. 2004; Della Vedova et al. 2005; Koseki et al. 2005). These phenomena also resemble the RNA silencing in a seed storage protein gene in rice, which is associated with a structural change in the gene region induced by mutagenesis (Kusaba et al. 2003), a case of RNA silencing in nontransgenic plants.

Visible RNA silencing as a tool to uncover regulatory mechanisms underlying the visible phenotypes

Because of its very high applicability, RNA silencing is suitable for knocking down a variety of genes. Large functional genomics projects that involve the systematic application of RNAi to plants are in progress (Hilson et al. 2004; McGinnis et al. 2005). In addition, RNA silencing of a particular gene is useful for analyzing biological phenomena, in particular those involving the effect of a difference in the mRNA level of the gene.

The following is an example of the use of VIGS as a tool to understand the regulatory mechanisms of pigmentation in soybean pubescence. VIGS of the *F3′H* gene, whose function is necessary for pigmentation of soybean pubescence, did not result in lack of pigmentation when plants were grown in normal greenhouse conditions, but resulted in lack of pigmentation when plants were grown in controlled conditions; the steady-state mRNA level of the *F3′H* gene was reduced to approximately 5% of that of greenhouse-grown plants (Nagamatsu et al. 2008). The VIGS in the controlled conditions resulted in a further decrease in the mRNA level, which led to the discovery of a threshold mRNA level of the *F3′H* gene associated with the occurrence of pigmented pubescence (Nagamatsu et al. 2008).

Such a threshold mRNA level for pigmentation can also be recognized in plant tissues that undergo RNA silencing, such as in soybean seed coats (Senda et al. 2004) and flower petals of transgenic (Metzlaff et al. 1997) and nontransgenic (Koseki et al. 2005) petunias, in which a low level of mRNA was detected, despite the occurrence of mRNA degradation that leads to nonpigmented phenotypes. An increase in the *CHS* mRNA level in flower petals with *CHS* co-suppression at 5% to 9% of the level in purple control petals resulted in a change from white to purple petals when *CHS* gene silencing was inhibited by a viral suppressor protein (Goto et al. 2007). These also serve as an example of the use of RNA silencing system to detect cellular regulatory mechanisms of pigmentation associated with the mRNA level of a gene.

A perspective on the application of RNA silencing in plant biotechnology

In an overview of the history behind our understanding of the principle of RNA silencing and its applications in genetic engineering, we need to recognize that RNA silencing manifesting as an altered visible phenotype has significantly contributed to our understanding of the phenomenon. Because the method to induce RNA silencing has already been established, we should be able to induce silencing of any genes by a routine method. However, much remains to be studied to establish RNA silencing as a more reliable tool in biotechnology.

One thing that needs to be examined may be the off-target effects of siRNA that might silence nontarget genes (reviewed by Mansoor et al. 2006; Small 2007). Such off-target effects have mainly been reported in large-scale analyses in animals, whereas no systematic studies have been completed in plants (Small 2007). Similarly, silencing of nontarget genes by “transitive silencing,” which involves the spread of RNA degradation of the target gene to related genes (Bleys et al. 2006a, b; Petersen and Albrechtsen 2005), might also be a problem. This phenomenon has been detected in plants, nematodes, and fungi, but what conditions the occurrence of this phenomenon still remains to be examined. In addition, the stability of the induced state of RNA silencing also needs to be examined extensively. A recent report indicated that epigenetic changes including methylation of transgene promoter occurred in a petunia line that has *CHS-A* co-suppression, and these changes interfere with the initiation of transgene transcription. These changes led to a reversion of the silenced phenotype, and the resultant plant has a flower color completely identical to that of nontransgenic plants (Kanazawa et al. 2007). A similar change in a transgene conferring drug resistance was observed in a long-term tobacco callus culture (Fojtova et al. 2003). It is tempting to speculate that the progression from PTGS to TGS seen in these transgenic plants reflects the genome’s natural gene silencing responses in which cells acquire a more stable tool to inactivate foreign DNA by a transition from PTGS to TGS.

These unexpected and/or destabilized silencing phenomena might limit the use of RNA silencing as a

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tool for plant biotechnology. Because these phenomena may occur in limited cells rather than in a whole plant, a sensitive method that allows detection of tissue-specific changes in gene expression is absolutely required to understand the mechanism(s) underlying the instability of RNA silencing. In this regard, there is an advantage in using plant endogenous genes whose changes in mRNA level are manifested as an altered visible phenotype. Most typically, the petunia *CHS* silencing system allows visual detection of RNA degradation in a very small number of cells. Similarly, the *PDS* gene has proved useful in visually and quantitatively assessing the effect of VIGS (Senthil-Kumar et al. 2007b).

Plant science has been playing a leading part in the study of RNA silencing. Reports of involvement of RNA silencing pathways in various biological phenomena in plants have been increasing rapidly. It is not surprising that an epoch-making discovery that answers these questions and improves efficacy of RNA silencing in biotechnology will come from unmasking natural mechanisms governing visible phenotypes in plants.

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References

Al-Kaff NS, Covey SN, Kreike MM, Page AM, Pinder R, Dale PJ (1998) Transcriptional and posttranscriptional plant gene silencing in response to a pathogen. *Science* 279: 2113–2115

Allen RS, Miller JA, Chitty JA, Fist AJ, Gerlach WL, Larkin PJ (2008) Metabolic engineering of morphinan alkaloids by over-expression and RNAi suppression of salutaridinol 7-O-acetyltransferase in opium poppy. *Plant Biotechnol J* 6: 22–30

Alvarez JP, Pekker I, Goldshmidt A, Blum E, Amsellem Z, Eshed Y (2006) Endogenous and synthetic microRNAs stimulate simultaneous, efficient, and localized regulation of multiple targets in diverse species. *Plant Cell* 18: 1134–1151

Atkinson RG, Bieleski LRF, Gleave AP, Janssen B-J, Morris BAM (1998) Post-transcriptional silencing of chalcone synthase in petunia using a geminivirus-based episomal vector. *Plant J* 15: 593–604

Baulcombe D (1996a) Mechanisms of pathogen-derived resistance to viruses in transgenic plants. *Plant Cell* 8: 1833–1844

Baulcombe D (1996b) RNA as a target and an initiator of post-transcriptional gene silencing in transgenic plants. *Plant Mol Biol* 32: 79–88

Baulcombe D (2004) RNA silencing in plants. *Nature* 431: 356–363

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

Bleys A, Van Houdt H, Depicker A (2006a) Down-regulation of endogenes mediated by a transitive silencing signal. *RNA* 12: 1633–1639

Bleys A, Vermeersch L, Van Houdt H, Depicker A (2006b) The frequency and efficiency of endogene suppression by transitive silencing signals is influenced by the length of sequence homology. *Plant Physiol* 142: 788–796

Boutet S, Vazquez F, Liu J, Béclin C, Fagard M, Gratias A, Morel JB, Crété P, Chen X, Vaucheret H (2003) *Arabidopsis HEN1*: a genetic link between endogenous miRNA controlling development and siRNA controlling transgene silencing and virus resistance. *Curr Biol* 13: 843–848

Brigneti G, Martín-Hernández AM, Jin H, Chen J, Baulcombe DC, Baker B, Jones JDG (2004) Virus-induced gene silencing in *Solanum* species. *Plant J* 39: 264–272

Brodersen P, Voinnet O (2006) The diversity of RNA silencing pathways in plants. *Trends Genet* 22: 268–280

Burch-Smith TM, Anderson JC, Martin GB, Dinesh-Kumar SP (2004) Applications and advantages of virus-induced gene silencing for gene function studies in plants. *Plant J* 39: 734–746

Burch-Smith TM, Schiff M, Liu Y, Dinesh-Kumar SP (2006) Efficient virus-induced gene silencing in Arabidopsis. *Plant Physiol* 142: 21–27

Burton RA, Gibeaut DM, Bacic A, Findlay K, Roberts K, Hamilton A, Baulcombe DC, Fincher GB (2000) Virus-induced silencing of a plant cellulose synthase gene. *Plant Cell* 12: 691–706

Cai XZ, Xu QF, Wang CC, Zheng Z (2006) Development of a virus-induced gene-silencing system for functional analysis of the *RPS2*-dependent resistance signalling pathways in *Arabidopsis*. *Plant Mol Biol* 62: 223–232

Chen J-C, Jiang C-Z, Gookin TE, Hunter DA, Clark DG, Reid MS (2004) Chalcone synthase as a reporter in virus-induced gene silencing studies of flower senescence. *Plant Mol Biol* 55: 521–530

Chung E, Seong E, Kim YC, Chung EJ, Oh SK, Lee S, Park JM, Joung YH, Choi D (2004) A method of high frequency virus-induced gene silencing in chili pepper (*Capsicum annuum* L. cv. Bukang). *Mol Cells* 17: 377–380

Cigan AM, Unger-Wallace E, Hang-Collet K (2005) Transcriptional gene silencing as a tool for uncovering gene function in maize. *Plant J* 43: 929–940

Cluster PD, O'Dell M, Metzlaff M, Flavell RB (1996) Details of T-DNA structural organization from a transgenic Petunia population exhibiting co-suppression. *Plant Mol Biol* 32: 1197–1203

Cogoni C, Macino G (1997) Isolation of quelling-defective (*qde*) mutants impaired in posttranscriptional transgene-induced gene silencing in *Neurospora crassa*. *Proc Natl Acad Sci USA* 94: 10233–10238

Constantin GD, Krath BN, MacFarlane SA, Nicolaisen M, Johansen IE, Lund OS (2004) Virus-induced gene silencing as a tool for functional genomics in a legume species. *Plant J* 40: 622–631

Covey SN, Al-Kaff NS, Lángara A, Turner DS (1997) Plants combat infection by gene silencing. *Nature* 385: 781–782

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Dalmay T, Hamilton A, Rudd S, Angell S, Baulcombe DC (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* 26: 543–553

Dalmay T, Horsefield R, Braunstein TH, Baulcombe DC (2001) SDE3 encodes an RNA helicase required for post-transcriptional gene silencing in *Arabidopsis*. *EMBO J* 17: 2069–2078

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

Deleris A, Gallego-Bartolome J, Bao J, Kasschau KD, Carrington JC, Voinnet O (2006) Hierarchical action and inhibition of plant Dicer-like proteins in antiviral defense. *Science* 313: 68–71

Della Vedova CB, Lorbiecke R, Kirsch H, Schulte MB, Scheets K, Borchert LM, Scheffler BE, Wienand U, Cone KC, Birchler JA (2005) The dominant inhibitory chalcone synthase allele *C2-Idf* (*Inhibitor diffuse*) from *Zea mays* (L.) acts via an endogenous RNA silencing mechanism. *Genetics* 170: 1989–2002

Ding XS, Schneider WL, Chaluvadi SR, Mian MA, Nelson RS (2006) Characterization of a Brome mosaic virus strain and its use as a vector for gene silencing in monocotyledonous hosts. *Mol Plant Microbe Interact* 19: 1229–1239

Dougherty WG, Lindbo JA, Smith HA, Parks TD, Swaney S, Proebsting WM (1994) RNA-mediated virus resistance in transgenic plants: exploitation of a cellular pathway possibly involved in RNA degradation. *Mol Plant Microbe Interact* 7: 544–552

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. *Nat Genet* 37: 1356–1360

Fagard M, Boutet S, Morel JB, Bellini C, Vaucheret H (2000) AGO1, QDE-2, and RDE-1 are related proteins required for post-transcriptional gene silencing in plants, quelling in fungi, and RNA interference in animals. *Proc Natl Acad Sci USA* 97: 11650–11654

Faivre-Rampant O, Gilroy EM, Hrubikova K, Hein I, Millam S, Loake GJ, Birch P, Taylor M, Lacomme C (2004) Potato virus X-induced gene silencing in leaves and tubers of potato. *Plant Physiol* 134: 1308–1316

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391: 806–811

Flavell RB (1994) Inactivation of gene expression in plants as a consequence of specific sequence duplication. *Proc Natl Acad Sci USA* 91: 3490–3496

Fofana IB, Sangare A, Collier R, Taylor C, Fauquet CM (2004) A geminivirus-induced gene silencing system for gene function validation in cassava. *Plant Mol Biol* 56: 613–624

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

Fu DQ, Zhu BZ, Zhu HL, Jiang WB, Luo YB (2005) Virus-induced gene silencing in tomato fruit. *Plant J* 43: 299–308

Fujii N, Inui T, Iwasa K, Morishige T, Sato F (2007) Knockdown of berberine bridge enzyme by RNAi accumulates (S)-reticuline and activates a silent pathway in cultured California poppy cells. *Transgenic Res* 16: 363–375

Goldbach R, Bucher E, Prins M (2003) Resistance mechanisms to plant viruses: an overview. *Virus Res* 92: 207–212

Goring DR, Thomson L, Rothstein SJ (1991) Transformation of a partial nopaline synthase gene into tobacco suppresses the expression of a resident wild-type gene. *Proc Natl Acad Sci USA* 88: 1170–1174

Gosselé V, Faché I, Meulewaeter F, Cornelissen M, Metzlaff M (2002) SVISS—a novel transient gene silencing system for gene function discovery and validation in tobacco plants. *Plant J* 32: 859–866

Goto K, Kobori T, Kosaka Y, Natsuaki T, Masuta C (2007) Characterization of silencing suppressor 2b of cucumber mosaic virus based on examination of its small RNA-binding abilities. *Plant Cell Physiol* 48: 1050–1060

Guo S, Kemphues KJ (1995) *par1*, a gene required for establishing polarity in *C. elegans* embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. *Cell* 81: 611–620

Hamilton AJ, Baulcombe DC (1999) A species of small antisence RNA in posttranscriptional gene silencing in plants. *Science* 286: 950–952

Heilersig BHJB, Loonen AEHM, Janssen EM, Wolters AMA, Visser RGF (2006) Efficiency of transcriptional gene silencing of GBSSI in potato depends on the promoter region that is used in an inverted repeat. *Mol Genet Genomics* 275: 437–449

Hein I, Barciszewska-Pacak M, Hrubikova K, Williamson S, Dinesen M, Soenderby IE, Sundar S, Jarmolowski A, Shirasu K, Lacomme C (2005) Virus-induced gene silencing-based functional characterization of genes associated with powdery mildew resistance in barley. *Plant Physiol* 138: 2155–2164

Helliwell CA, Waterhouse PM (2005) Constructs and methods for hairpin RNA-mediated gene silencing in plants. *Methods Enzymol* 392: 24–35

Hileman LC, Drea S, de Martino G., Litt A, Irish VF (2005) Virus-induced gene silencing is an effective tool for assaying gene function in the basal eudicot species *Papaver somniferum* (opium poppy). *Plant J* 44: 334–341

Hilson P, Allemeersch J, Altmann T, Aubourg S, Avon A, Beynon J, Bhalerao RP, Bitton F, Caboche M, Cannoot B, Chardakov V, Cognet-Holliger C, Colot V, Crowe M, Darimont C, Durinck S, Eickhoff H, de Longevialle AF, Farmer EE, Grant M, Kuiper MT, Lehrach H, Léon C, Leyva A, Lundeberg J, Lurin C, Moreau Y, Nietfeld W, Paz-Ares J, Reymond P, Rouzé P, Sandberg G, Segura MD, Serizet C, Tabrett A, Taconnat L, Thareau V, Van Hummelen P, Vercruysse S, Vuylsteke M, Weingartner M, Weisbeek PJ, Wirta V, Wittink FR, Zabeau M, Small I (2004) Versatile gene-specific sequence tags for Arabidopsis functional genomics: transcript profiling and reverse genetics applications. *Genome Res* 14: 2176–2189

Holzberg S, Brosio P, Gross C, Pogue GP (2002) Barley stripe mosaic virus-induced gene silencing in a monocot plant. *Plant J* 30: 315–327

Hori K, Takizawa M, Watanabe Y (2004) Use of an attenuated strain of tobamovirus for early detection of virus-induced gene silencing. *Plant Biotechnol* 21: 135–142

Houmard NM, Mainville JL, Bonin CP, Huang S, Luethy MH, Malvar TM (2007) High-lysine corn generated by endosperm-specific suppression of lysine catabolism using RNAi. *Plant Biotechnol J* 5: 605–614

Jones L, Hamilton AJ, Voinnet O, Thomas CL, Maule AJ, Baulcombe DC (1999) RNA-DNA interactions and DNA methylation in post-transcriptional gene silencing. *Plant Cell* 11: 2291–2301

Jones L, Ratcliff F, Baulcombe DC (2001) RNA-directed

Copyright © 2008 The Japanese Society for Plant Cell and Molecular Biology

transcriptional gene silencing in plants can be inherited independently of the RNA trigger and requires Met1 for maintenance. *Curr Biol* 11: 747–757

Jorgensen RA (1995) Cosuppression, flower color patterns, and metastable gene expression states. *Science* 268: 686–691

Kanazawa A, O'Dell M, Hellens RP (2007) Epigenetic inactivation of *chalcone synthase-A* transgene transcription in petunia leads to a reversion of the post-transcriptional gene silencing phenotype. *Plant Cell Physiol* 48: 638–647

Kasai A, Kasai K, Yumoto S, Senda M (2007) Structural features of GmIRCHS, candidate of the I gene inhibiting seed coat pigmentation in soybean: implications for inducing endogenous RNA silencing of chalcone synthase genes. *Plant Mol Biol* 64: 467–479

Kjemtrup S, Sampson KS, Peele CG, Nguyen LV, Conkling MA, Thompson WF, Robertson D (1998) Gene silencing from plant DNA carried by a Geminivirus. *Plant J* 14: 91–100

Koseki M, Goto K, Masuta C, Kanazawa A (2005) The star-type color pattern in *Petunia hybrida* 'Red Star' flowers is induced by sequence-specific degradation of chalcone synthase RNA. *Plant Cell Physiol* 46: 1879–1883

Kumagai MH, Donson J, della-Cioppa G, Harvey D, Hanley K, Grill LK (1995) Cytoplasmic inhibition of carotenoid biosynthesis with virus-derived RNA. *Proc Natl Acad Sci USA* 92: 1679–1683

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

Lacomme C, Hrubikova K, Hein I (2003) Enhancement of virus-induced gene silencing through viral-based production of inverted-repeats. *Plant J* 34: 543–553

Lindbo JA, Silva-Rosales L, Proebsting WM, Dougherty WG (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

Liu Y, Schiff M, Dinesh-Kumar SP (2002a) Virus-induced gene silencing in tomato. *Plant J* 31: 777–786

Liu Y, Schiff M, Marathe R, Dinesh-Kumar SP (2002b) Tobacco *Rar1*, *EDS1* and *NPR1*/*NIM1* like genes are required for *N*-mediated resistance to tobacco mosaic virus. *Plant J* 30: 415–429

Lu R, Martin-Hernandez AM, Peart JR, Malcuit I, Baulcombe DC (2003) Virus-induced gene silencing in plants. *Methods* 30: 296–303

Mallory AC, Vaucheret H (2006) Functions of microRNAs and related small RNAs in plants. *Nat Genet* 38 Suppl: S31–S36

Mansoor S, Amin I, Hussain M, Zafar Y, Briddon RW (2006) Engineering novel traits in plants through RNA interference. *Trends Plant Sci* 11: 559–565

Matzke MA, Birchler JA (2005) RNAi-mediated pathways in the nucleus. *Nat Rev Genet* 6: 24–35

Matzke M, Primig M, Trnovsky J, Matzke A (1989) Reversible methylation and inactivation of marker genes in sequentially transformed plants. *EMBO J* 8: 643–649

Matzke M, Matzke AJM, Kooter JM (2001) RNA: guiding gene silencing. *Science* 293: 1080–1083

Matzke M, Aufsatz W, Kanno T, Daxinger L, Papp I, Mette MF, Matzke AJM (2004) Genetic analysis of RNA-mediated transcriptional gene silencing. *Biochim Biophys Acta* 1677: 129–141

McGinnis K, Chandler V, Cone K, Kaeppler H, Kaeppler S, Kerschen A, Pikaard C, Richards E, Sidorenko L, Smith T, Springer N, Wulan T (2005) Transgene-induced RNA interference as a tool for plant functional genomics. *Methods Enzymol* 392: 1–24

Mette MF, van der Winden J, Matzke MA, Matzke AJM (1999) Production of aberrant promoter transcripts contributes to methylation and silencing of unlinked homologous promoters *in trans*. *EMBO J* 18: 241–248

Mette MF, Aufsatz W, van der Winden J, Matzke MA, Matzke AJM (2000) Transcriptional silencing and promoter methylation triggered by double-stranded RNA. *EMBO J* 19: 5194–5201

Metzlaff M, O'Dell M, Cluster PD, Fravell RB (1997) RNA-mediated degradation and chalcone synthase A silencing in petunia. *Cell* 88: 845–854

Molnár A, Csorba T, Lakatos L, Váralleyay É, Lacomme C, Burgyán J (2005) Plant virus-derived small interfering RNAs originate predominantly from highly structured single-stranded viral RNAs. *J Virol* 79: 7812–7818

Morel JB, Godon C, Mourrain P, Béclin C, Boutet S, Feuerbach F, Proux F, Vaucheret H (2002) Fertile hypomorphic *ARGONAUTE* (*ago1*) mutants impaired in post-transcriptional gene silencing and virus resistance. *Plant Cell* 14: 629–639

Moritoh S, Miki D, Akiyama M, Kawahara M, Izawa T, Maki H, Shimamoto K (2005) RNAi-mediated silencing of OsGEN-L (OsGEN-like), a new member of the RAD2/XPG nuclease family, causes male sterility by defect of microspore development in rice. *Plant Cell Physiol* 46: 699–715

Mourrain P, Béclin C, Elmayan T, Feuerbach F, Godon C, Morel JB, Jouette D, Lacombe AM, Nikic S, Picault N, Rémoué K, Sanial M, Vo TA, Vaucheret H (2000) *Arabidopsis SGS2* and *SGS3* genes are required for posttranscriptional gene silencing and natural virus resistance. *Cell* 26: 533–542

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

Muskens MWM, Vissers APA, Mol JNM, Kooter JM (2000) Role of inverted DNA repeats in transcriptional and post-transcriptional gene silencing. *Plant Mol Biol* 43: 243–260

Nagamatsu A, Masuta C, Senda M, Matsuura H, Kasai A, Hong JS, Kitamura K, Abe J, Kanazawa A (2007) Functional analysis of soybean genes involved in flavonoid biosynthesis by virus-induced gene silencing. *Plant Biotechnol J* 5: 778–790

Nagamatsu A, Masuta C, Matsuura H, Kitamura K, Abe J, Kanazawa A (2008) Down-regulation of flavonoid 3′-hydroxylase gene expression by virus-induced gene silencing in soybean reveals the presence of a threshold mRNA level associated with pigmentation in pubescence. *J Plant Physiol* (in press)

Napoli C, Lemieux C, Jorgensen R (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

Niu QW, Lin SS, Reyes JL, Chen KC, Wu HW, Yeh SD, Chua NH (2006) Expression of artificial microRNAs in transgenic *Arabidopsis thaliana* confers virus resistance. *Nat Biotechnol* 24: 1420–1428

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

Okano Y, Miki D, Shimamoto K (2008) Small interfering RNA

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(siRNA) targeting of endogenous promoters induces DNA methylation, but not necessarily gene silencing, in rice. *Plant J* 53: 65–77

Ossowski S, Schwab R, Weigel D (2008) Gene silencing in plants using artificial microRNAs and other small RNAs. *Plant J* 53: 674–690

Otagaki S, Arai M, Takahashi A, Goto K, Hong JS, Masuta C, Kanazawa A (2006) Rapid induction of transcriptional and post-transcriptional gene silencing using a novel *Cucumber mosaic virus* vector. *Plant Biotechnol* 23: 259–265

Page DR, Grossniklaus U (2002) The art and design of genetic screens: *Arabidopsis thaliana*. *Nat Rev Genet* 3: 124–136

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

Pal-Bhadra M, Bhadra U, Birchler JA (1997) Cosuppression in Drosophila: gene silencing of *Alcohol dehydrogenase* by *white-Adh* transgenes is *Polycomb* dependent. *Cell* 90: 479–490

Park Y-D, Moscone EA, Iglesis VA, Vaucheret H, Matzke AJM, Matzke MA (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

Peele C, Jordan CV, Muangsan N, Turnage M, Egelkrout E, Eagle P, Hanley-Bowdoin L, Robertson D (2001) Silencing of a meristematic gene using geminivirus-derived vectors. *Plant J* 27: 357–366

Peragine A, Yoshikawa M, Wu G, Albrecht HL, Poethig RS (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

Petersen BO, Albrechtsen M (2005) Evidence implying only unprimed RdRP activity during transitive gene silencing in plants. *Plant Mol Biol* 58: 575–583

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

Qiu W, Park JW, Scholthof HB (2002) Tombusvirus P19-mediated suppression of virus-induced gene silencing is controlled by genetic and dosage features that influence pathogenicity. *Mol Plant Microbe Interact* 15: 269–280

Ratcliff F, Martin-Hernandez AM, Baulcombe DC (2001) Tobacco rattle virus as a vector for analysis of gene function by silencing. *Plant J* 25: 237–245

Romeis T, Ludwig AA, Martin R, Jones JD (2001) Calcium-dependent protein kinases play an essential role in a plant defence response. *EMBO J* 20: 5556–5567

Ruiz MT, Voinnet O, Baulcombe DC (1998) Initiation and maintenance of virus-induced gene silencing. *Plant Cell* 10: 937–946

Ryu CM, Anand A, Kang L, Mysore KS (2004) Agrodrench: a novel and effective agroinoculation method for virus-induced gene silencing in roots and diverse Solanaceous species. *Plant J* 40: 322–331

Saitoh H, Terauchi R (2002) Virus-induced silencing of *FtsH* gene in *Nicotiana benthamiana* causes a striking bleached leaf phenotype. *Genes Genet Syst* 77: 335–340

Schwab R, Ossowski S, Riester M, Warthmann N, Weigel D (2006) Highly specific gene silencing by artificial microRNAs in *Arabidopsis*. *Plant Cell* 18: 1121–1133

Scofield SR, Huang L, Brandt AS, Gill BS (2005) Development of a virus-induced gene-silencing system for hexaploid wheat and its use in functional analysis of the Lr21-mediated leaf rust resistance pathway. *Plant Physiol* 138: 2165–2173

Senda M, Masuta C, Ohnishi S, Goto K, Kasai A, Sano T, Hong JS, MacFarlane S (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

Senthil-Kumar M, Govind G, Kang L, Mysore KS, Udayakumar M (2007a) Functional characterization of *Nicotiana benthamiana* homologs of peanut water deficit-induced genes by virus-induced gene silencing. *Planta* 225: 523–539

Senthil-Kumar M, Hema R, Anand A, Kang L, Udayakumar M, Mysore KS (2007b) A systematic study to determine the extent of gene silencing in *Nicotiana benthamiana* and other Solanaceae species when heterologous gene sequences are used for virus-induced gene silencing. *New Phytol* 176: 782–791

Sharma PC, Ito A, Shimizu T, Terauchi R, Kamoun S, Saitoh H (2003) Virus-induced silencing of WIPK and SIPK genes reduces resistance to a bacterial pathogen, but has no effect on the INF1-induced hypersensitive response (HR) in *Nicotiana benthamiana*. *Mol Genet Genomics* 269: 583–591

Sijen T, Wellink J, Hiriart J-B, van Kammen A (1996) RNA-mediated virus resistance: role of repeated transgenes and delineation of targeted regions. *Plant Cell* 8: 2277–2294

Sijen T, Vijn I, Rebocho A, van Blockland R, Roelofs D, Mol JNM, Kooter JM (2001) Transcriptional and posttranscriptional gene silencing are mechanistically related. *Curr Biol* 11: 436–440

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

Small I (2007) RNAi for revealing and engineering plant gene functions. *Curr Opin Biotechnol* 18: 148–153

Smith CJS, Watson CF, Bird CR, Ray J, Schuch W, Grierson D (1990) Expression of a truncated tomato polygalacturonase gene inhibits expression of the endogenous gene in transgenic plants. *Mol Gen Genet* 224: 447–481

Smith HA, Swaney SL, Parks TD, Wernsman EA, Dougherty WG. (1994) Transgenic plant virus resistance mediated by untranslatable sense RNAs: expression, regulation, and fate of nonessential RNAs. *Plant Cell* 6: 1441–1453

Smith NA, Singh SP, Wang MB, Stoutjesdijk PA, Green AG, Waterhouse PM (2000) Total silencing by intron-spliced hairpin RNAs. *Nature* 407: 319–320

Stam M, Viterbo A, Mol JNM, Kooter JM (1998) Position-dependent methylation and transcriptional silencing of transgenes in inverted repeats: implications for posttranscriptional silencing of homologous host genes in plants. *Mol Cell Biol* 18: 6165–6177

Sunilkumar G, Campbell LM, Puckhaber L, Stipanovic RD, Rathore KS (2006) Engineering cottonseed for use in human nutrition by tissue-specific reduction of toxic gossypol. *Proc Natl Acad Sci USA* 103: 18054–18059

Takizawa M, Hori K, Inai K, Takase H, Hashimoto T, Watanabe Y (2007) A virus-induced gene silencing approach for the suppression of nicotine content in *Nicotiana benthamiana*. *Plant Biotechnol* 24: 295–300

Tang G, Galili G (2004) Using RNAi to improve plant nutritional value: from mechanism to application. *Trends Biotechnol* 22: 463–469

Tao X, Zhou X (2004) A modified viral satellite DNA that suppresses gene expression in plants. *Plant J* 38: 850–860

Turnage MA, Muangsan N, Peele CG, Robertson D (2002)

Copyright © 2008 The Japanese Society for Plant Cell and Molecular Biology

Geminivirus-based vectors for gene silencing in *Arabidopsis*. *Plant J* 30: 107–114

Valentine T, Shaw J, Blok VC, Phillips MS, Oparka KJ, Lacomme C (2004) Efficient virus-induced gene silencing in roots using a modified tobacco rattle virus vector. *Plant Physiol* 136: 3999–4009

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

van der Krol AR, Mur LA, Beld M, Mol JNM, Stuitje AR (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

Vaucheret H (1993) Identification of a general silencer for 19S and 35S promoters in a transgenic tobacco plant: 90 bp of homology in the promoter sequences are sufficient for trans-inactivation. *C R Acad Sci Paris* 316: 1471–1483

Vaucheret H (2006) Post-transcriptional small RNA pathways in plants: mechanisms and regulations. *Genes Dev* 20: 759–771

Vazquez F, Vaucheret H, Rajagopalan R, Lepers C, Gasciolli V, Mallory AC, Hilbert JL, Bartel DP, Crété P (2004) Endogenous trans-acting siRNAs regulate the accumulation of *Arabidopsis* mRNAs. *Mol Cell* 16: 69–79

Voinnet O (2002) RNA silencing: small RNAs as ubiquitous regulators of gene expression. *Curr Opin Plant Biol* 5: 444–451

Voinnet O, Baulcombe DC (1997) Systemic signalling in gene silencing. *Nature* 389: 553

Voinnet O, Rivas S, Mestre P, Baulcombe D (2003) An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. *Plant J* 33: 949–956

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

Waterhouse PM, Graham MW, 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

Wesley SV, Helliwell CA, Smith NA, Wang MB, Rouse DT, Liu Q, Gooding PS, Singh SP, Abbott D, Stoutjesdijk PA, Robinson SP, Gleave AP, Green AG, Waterhouse PM (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. *Plant J* 27: 581–590

Wilson TMA (1993) Strategies to protect crop plants against viruses: pathogen-derived resistance blossoms. *Proc Natl Acad Sci USA* 90: 3134–3141

Yang S-J, Carter SA, Cole AB, Cheng N-H, Nelson RS (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

Zhang C, Ghabrial SA (2006) Development of *Bean pod mottle virus*–based vectors for stable protein expression and sequence-specific virus-induced gene silencing in soybean. *Virology* 344: 401–411

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