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Review

RNAi and functional genomics

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Abstract Various genome projects have recently provided abundant data on the genomes of different organisms, from simple microbes to higher plants and animals. More tools are needed to characterize the function of these many genes. RNAi offers many advantages regarding the down-regulation of gene expression. To increase the effectiveness of RNAi, novel RNAi methods (transient RNAi, differential RNAi (dRNAi), comprehensive RNAi with simple construction, quantitatively regulated RNAi, etc.) have been developed for functional genomics in plant. These methods have been used to reveal the functions of many genes and functional networks in genomes of higher plants. The further development of a gene-substitution method based on dRNAi could provide a new tool for the characterization of plant gene functions using a more rational approach. RNAi has also been shown to be useful in metabolic engineering. Detailed characterization of the mechanism of RNAi could also provide the molecular basis for the next generation of gene engineering.

Key words: Differential RNAi (dRNAi), DNA methylation, functional genomics, metabolic engineering, non-model plants, rice, transient RNAi, transposon, virus suppressors.

We now stand at the threshold of the post-genome era, in which whole plant functions will be characterized based on the rapidly growing body of genome data (Gura 2000), considering that the genome sequences of *Arabidopsis* (EU Arabidopsis Chromosome 3 Sequence Consortium 2000; Kazusa Research Institute et al. 2000) and rice (International Rice Genome Sequencing Project 2005) have been completely elucidated. While whole genome sequences are the basis of further characterization, the identification of gene functions is needed for further functional genomics based on genome sequences. In addition to comprehensive expression analysis with a DNA microarray to characterize gene expression, the ectopic expression of a target gene in heterologous host cells such as *Escherichia coli*, yeast or host plants is useful for understanding gene functions. Whereas such an over-expression system under a constitutive or inducible promoter is highly effective, gene silencing techniques are also very important for characterizing gene function in target cells since the disruption of gene expression often shows a more critical phenotype. To date, gene knockout mutants, antisense RNA, ribozyme and co-suppression have been used for the down-regulation of gene expression, but RNA interference (RNAi), double-stranded (ds)RNA-based degradation of a target mRNA has shown much higher potential for such a purpose, since short interfering RNA of 21–23 nucleotides has been shown to be quite effective for gene silencing in mammalian cells (Tuschl 2003; Hannon and Rossi 2004; Mello and Conte 2004).

Whereas dsRNA-based RNA silencing is fairly common in plants, actual technological improvements in RNAi, especially for the comprehensive analysis of the whole genome, in plant cells are still limited. Thus, we have tried to establish simpler and more efficient systems for using RNAi for functional genomics in plants. This paper summarizes some of our progress in a project on "RNAi and Functional Genomics" supported by the JSPS. In this review, the term "RNAi" is used to mean RNA silencing, while the latter term is used more often

Abbreviations: BBE, berberine bridge enzyme; dRNAi, differential RNA interference; dsRNA, double-stranded RNA; EST, expression sequenced tag; miRNA, microRNA; OEC, oxygen-evolving complex; PSII, photosystem II; PTGS, post-transcriptional gene silencing; RNAi, RNA interference; RdRp, RNA-dependent RNA polymerase; RISC, RNA-induced silencing complex; RT-PCR, reverse transcription-polymerase chain reaction; siRNA, short interfering RNA; SMT, scoulerine 9-*O*-methyltransferase; TGS, transcriptional gene silencing; UTR, untranslated region.

This project "JSPS-RFTF00L01606" was conducted by a collaborative research group that consisted of Drs. E Fukusaki and CI An of the Graduate School of Engineering, Osaka University, Drs. K Ito and F Tanno of the Graduate School of Science and Technology, Niigata University, Drs. K Shimamoto T Izawa and M Isshiki of the Graduate School of Bioscience, Nara Institute of Science and Technology, Dr. T Kakutani of the Genetic Institute, Drs. K Mise T Okuno and Y Nanjo of the Graduate School of Agriculture, Kyoto University, and Drs. K Yamato, K Ifuku, T Nakamura, Y Yamamoto, and H Minami of the Graduate School of Biostudies, Kyoto University. This review is based on their research achievements and an outline of their progress with a list of publications will be reported at the JSPS website. Due to space limitations, this summary describes only some of their achievements. This author sincerely appreciates their valuable contributions and apologizes that many important findings could not be cited in this review.
This article can be found at http://www.jspcmb.jp/

in plant sciences.

Molecular mechanism of RNAi

While gene silencing based on RNAi (RNA interference) was first reported to be induced by the introduction of double-stranded (ds)RNA in *Caenorhabditis elegans* cells in 1998 (Fire et al. 1998), dsRNA-dependent RNA silencing is a rather common phenomenon in plants; e.g., the co-suppression of endogenous gene expression by the introduction of the ectopic expression of a sense gene has been reported (Que and Jorgensen 1998). We now realize that such co-suppression or some virus-resistance phenomenon in plants can be based on RNA-degradation via dsRNA (Baulcombe 2004). In fact, such mRNA-degradation based on dsRNA, i.e., RNAi (or RNA silencing), is a common genetic mechanism for controlling gene expression in both plants and animals (Baulcombe 2004; Meister and Tuschl 2004).

Figure 1 outlines the molecular mechanism in RNAi. In brief, dsRNA molecules are generated through aberrant gene expression, virus infection or a tandem repeat sequence due to the insertion of a transposon. The resulting dsRNA molecules are digested into 21–25 nucleotide-long siRNA by Dicer (an RNaseIII-like RNase). This siRNA acts as a template for the targeted degradation of mRNA in RISC (RNA-induced silencing complex). So far, many molecular components in the RNAi machinery have been characterized and secondary RNAi based on RNA-dependent RNA polymerase (RdRp) has been reported in some animals such *C. elegans* (Nishikura 2001). In general, RNAi has two potential mechanisms for down-regulating gene expression; post-transcriptional gene silencing (PTGS) through either mRNA degradation as described above or translational arrest, and transcriptional gene silencing (TGS) via the methylation of DNA. While we mainly focused on the former phenomenon, TGS is also important in the regulation of endogenous genes and the application of RNAi.

In brief, RNAi has great potential as a tool for down-regulating gene expression (Table 1). RNAi induced by the introduction (expression) of dsRNA into target cells induced much stronger and selective gene silencing. For example, very simple methods, such as the introduction of artificially synthesized dsRNA into cells by dipping, microinjection or feeding of *E. coli* with dsRNA expression vector, can be applied in the case of *C. elegans*, the most sensitive host for RNAi. Transient RNAi has also been successfully used for the

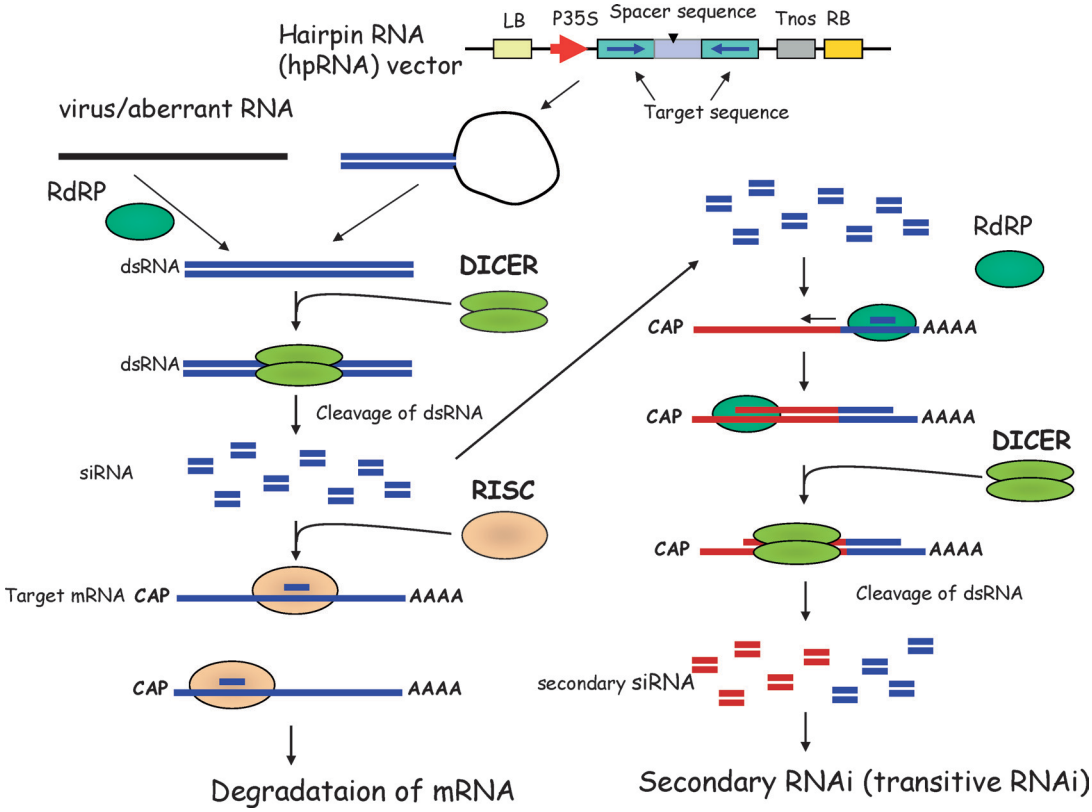

Figure 1. The current model of RNA-mediated gene silencing in plants. Double-stranded RNA (dsRNA) generated through aberrant gene expression from a foreign gene, virus infection or tandem repeat sequence due to insertion of a transposon/retrotransposon is digested into 21–25 nucleotide-long short interfering RNA (siRNA) by Dicer (an RNaseIII-like RNase). This siRNA functions as a template for the targeted degradation of mRNA in RISC (RNA-induced silencing complex). SiRNA also acts as the primer for RNA-dependent RNA polymerase (RdRp) to amplify the secondary dsRNA, although some differences between endogeneous and foreign genes have been found for secondary RNAi.

Table 1.

Advantages of RNAi
1) High sequence-specific gene silencing
2) Highly efficient gene silencing
3) Possibility of gene silencing with short dsRNA such as 21–23-nucleotide-long siRNA
4) Systemic spreading of gene silencing
5) Transitivity of gene silencing due to the secondary generation of dsRNA*
Weaknesses
1) Non-specific (off-target) gene silencing**
2) Suppression of gene silencing

* observed in *C. elegans* and some plants. Note that systemic/transitive RNAi has mainly been observed for introduced foreign genes and not for endogenous genes in plants. ** In comparison with plant cells, animal cells showed low stringency for RNAi, especially for microRNA. This is less likely for plant cells.

Table 2. Advantages and weaknesses in stable and transient RNAi in plants (modified from Waterhouse and Helliwell 2003).

Methods	Advantages	Weaknesses
Stable RNAi	No limitation of gene silencing period	Difficulty of high-throughput screening
Hairpin RNA vector	Suitable for all transformable plants	Requires transformation
	Heritable to next generation	Requires construction of vector
	Tissue-specific or inducible silencing	
Virus amplicon	Broad range of suitable hosts	Requires construction of vector
	Tissue-specific or inducible silencing, less damage due to virus infection	
Transient RNAi	Rapid	Limitation of gene silencing period
Direct transfer		Only for the phenotype which can be analyzed in single cells
Hairpin RNAi vector	Tissue-specific or inducible silencing	Tedious vector construction
Synthetic dsRNA	Easy preparation for high-thoughput analysis	
+particle gun	No limitation of plant species	Requires equipment, low transformation efficiency
+electroporation/ polyethyleneglycol	High transformation efficiency	Requires protoplast preparation
Agroinfection		
(with Hairpin RNAi vector)	Easy and low cost	Limitation of host plant species, e.g.
	Tissue-specific or inducible silencing may be possible	*Nicotiana benthamiana*, tedious vector construction
VIGS**	Easy and high cost	Limitation of host plant species, e.g.
	Whole plant analysis possible via systemic silencing	*Nicotiana benthamiana*, requires vector construction
	High-throughput analysis possible	Possible side effect due to virus amplification

* Transient infection of plant tissues with *Agrobacterium* having Ti plasmid vector.
** Virus-induced gene silencing; infection with virus vector which can self-replicate in host plants.

comprehensive analysis of gene functions. Especially, the finding of 21–25 nucleotide-long siRNA, as the active ingredient for RNAi machinery, has enabled it to be used for RNAi in mammalian cells and expanded the scope of RNAi (Elbashir et al. 2001; Tuschl 2003; Hannon and Rossi 2004; Mello and Conte 2004).

Development of transient RNAi in plant systems

Although RNAi technology was established in plants and used for the characterization of gene functions at the beginning of this project (around year 2000), the development of a transient RNAi system for plants was delayed due to difficulties in the development of a suitable transformation system with dsRNA (see Table 2). A low transformation efficiency in a transient assay, the difficulty of introducing dsRNA into the cytoplasm/nucleus by physical means in the presence of a large vacuole and a rather long time span for observing the phenotype have hindered the establishment of an efficient transient system in plants. On the other hand, the construction of expression vectors for RNAi is especially tricky due to the nature of the inverted repeat structure, whereas stable transformation is important for determining the function of plant genes.

Several attempts have been made to establish transient systems in plants (Waterhouse and Helliwell 2003) (Table 2); e.g., the introduction of artificially synthesized dsRNA, virus-amplicon via *Agrobacterium*, and/or VIGS (virus-induced gene silencing) using virus vectors have been developed. Especially, the VIGS vector system, developed by Dr. Baulcombe's group at the John Innes Center, is the most efficient transient system developed thus far. VIGS consists of an *Agrobacterium* gene expression system to produce dsRNA via virus-RNA-dependent RNA polymerase of potato virus X (PVX) or tobacco rattle virus (TRV), and induces gene silencing in the whole plant (Baulcombe 1999). Since VIGS can be intensified through the amplification of virus after

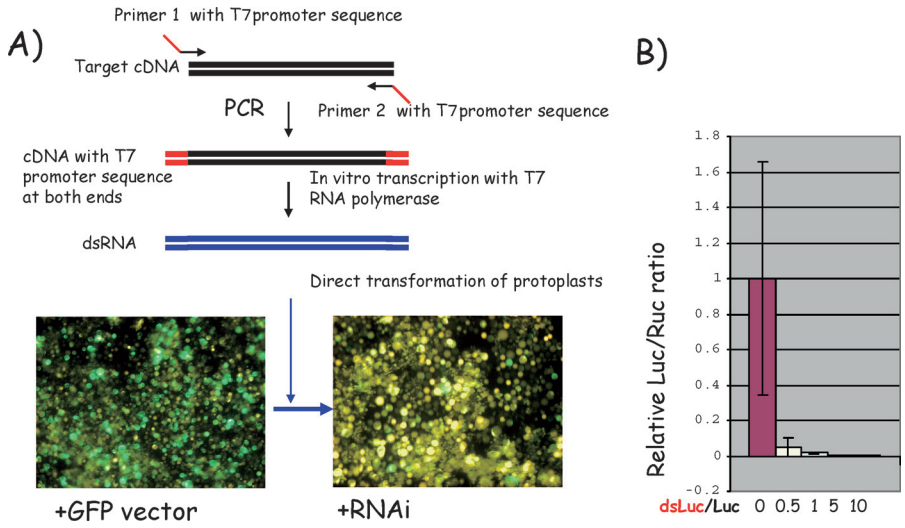

Figure 2. Transient RNAi in *Coptis japonica* protoplast. dsRNA was synthesized in vitro using T7 RNA polymerase and PCR products with T7 promoter sequences at both ends. When *C. japonica* protoplasts were transformed via polyethyleneglycol with the luciferase reporter gene (1 μg LUC expression plasmid) as a model, we found that the addition of 0.5 μg dsRNA of LUC to about 100,000 *C. japonica* protoplasts dramatically reduced the relative luminescence (Dubouzet et al. 2005). A high transformation efficiency (more than 90%) and efficient gene silencing in *C. japonica* protoplasts were also observed when GFP expression vector was used as a reporter.

infection, the gene silencing effects are very strong and effective. However, few host plant species are suitable for VIGS, since VIGS is dependent on the host range of virus vectors used (Waterhouse and Helliwell 2003).

In comparison with VIGS, the introduction of dsRNA-expression vector via *Agrobacterium* infection (Agroinfection; Johansen and Carrington 2001), or the direct introduction of dsRNA-expression vector or dsRNA/siRNA themselves can be applied to a much wider range of plant species (Schweizer et al. 2000; Akashi et al. 2001; Wesley et al. 2001; Vanitharani et al. 2003). However, one disadvantage of these systems is the limitation on the amount of dsRNA introduced/produced; i.e., there is no amplification of dsRNA. The construction of dsRNA expression vectors, the preparation of dsRNA, and the evaluation of RNAi effects within a restricted time span have also limited the application of this approach. In fact, we have often encountered the instability of a typical hairpin structure in an RNAi vector during construction, even when we have constructed RNAi vectors using Gateway systems (Wesley et al. 2001). Thus, a comprehensive analysis based on RNAi expression vectors could be rather restricted due to the vectors used. Therefore, we have tried to develop a new transient system to easily evaluate gene function in plant cells (An et al. 2003, 2005; Dubouzet et al. 2005).

In our project, Eiichiro Fukusaki, Fumihiko Sato and their respective groups decided to use a direct gene transfer system with dsRNA in protoplasts after several empirical trials with whole plant systems. RNAi in plant protoplasts was first demonstrated by Akashi et al. (2001). However, they used a dsRNA expression plasmid to silence the expression of the luciferase reporter gene, when they observed silencing of luciferase gene expression in tobacco BY-2 protoplasts within 24 h of electroporation. To simplify the construction of the expression vector, we used a system in which dsRNA is synthesized in vitro (Carthew and Kennerdell: http://www.bioexchange.com/tools/protocol detail.cfm?protocol id=31#MMB101) using T7 RNA polymerase and PCR products with T7 promoter sequences at both ends. This synthesized dsRNA was shown to be effective for the efficient RNAi of the LUC reporter gene in *Arabidopsis thaliana* protoplasts (An et al. 2003). Sato's group then developed an efficient gene-silencing system for the endogenous gene in *Coptis japonica* 156-SMT protoplasts as a model (Dubouzet et al. 2005) (Figure 2).

When *C. japonica* protoplasts were transformed via polyethyleneglycol (Sheen, http://genetics.mgh.harvard.edu/sheenweb/) with the luciferase reporter gene (1 μg LUC expression plasmid) as a model, we found that the addition of 0.5 μg dsRNA of LUC to about 100,000 *C. japonica* protoplasts dramatically reduced the relative luminescence; the activity that remained at a dose of more than 1.0 μg dsRNA reached a level of less than 2% of that in the control without dsRNA (Dubouzet et al. 2005). This level of silencing was not achieved in previous studies that used protoplasts from *Arabidopsis* or tobacco BY-2 (Akashi et al. 2001; An et al. 2003). A high transformation efficiency (more than 90%) and efficient gene silencing in *C. japonica* protoplasts were also observed when the GFP expression vector was used as a reporter, clearly indicating that *C. japonica* cells are quite suitable for RNAi research. These results are comparable to those for RNAi in animal cells and

provide a useful basis for the comprehensive analysis of gene functions, when applied to endogenous genes.

Further experiments to examine the effect of dsRNA on an endogeneous gene revealed that the scoulerine *O*-methyltransferase (SMT) transcript level was decreased by dsRNAs prepared from different portions (5′ or 3′) of the coding sequence of SMT (Dubouzet et al. 2005). Whereas the initial RNAi effect for the endogenous SMT was relatively weak, an increase in dsRNA to 50 μg/10^6 protoplasts increased the RNAi effect, indicating that the RNAi effect is dose-dependent. Thus, in comparison with the model RNAi experiment for luciferase, the amount of dsRNA required for endogenous SMT appears to be slightly high. The preparation of dsRNA from different parts of SMT cDNA has indicated the robustness of the silencing ability of dsRNA and the laxity of the design requirements for silencing of specific genes. Thus, 230-bp dsRNA derived from any segment of the target gene will probably be as active as longer dsRNAs or specifically constructed "designer" dsRNAs.

dsRNA was effective for 24–72 h after treatment and led to a detectable decrease in the SMT protein level, whereas the decrease in protein was about 50% and much less than the decrease in transcripts. When we examined RNA silencing for other endogenous *O*- or *N*-methyltransferase using dsRNA of about 230–231 bp, including the relatively conserved methyltransferase domain, some dsRNA such as norcoclaurine 6-*O*-methyltransferase showed moderate cross-silencing effects on related genes such as 3′-hydroxy-*N*-methylcoclaurine 4′-*O*-methyltransferase and SMT with close sequence homology. This cross-silencing between dsRNA and non-target genes was found among methyltransferases in berberine biosynthesis, while an approximately 20-bp identical stretch was also found (Dubouzet et al. 2005). Whereas we could not determine the sequence preference of RNAi in *C. japonica* protoplasts, the data suggest that dsRNA of at least 100 bp in length would have sufficient sequence information for RNAi, and multiple genes (at most 5 genes in our experiments) can be silenced simultaneously. Our results further confirm the utility of gene silencing based on in vitro-synthesized dsRNA for studying the function and behavior of endogenous genes.

One important finding was the enhanced expression of SMT in *C. japonica* protoplasts. This gene activation helped us to evaluate the function of genes involved in berberine biosynthesis using RNAi, since we could neglect the basal transcripts and detect the silencing effect more clearly. Thus, we analyzed potential transcriptional factor candidate genes isolated from an expression sequenced tag (EST) library of high berberine-producing *C. japonica* cells using this transient RNAi system and monitored the transcript level of down-stream biosynthetic genes. After screening about 50 candidates, we successfully identified several trans-factors, which are involved in the comprehensive regulation of isoquinoline alkaloid biosynthesis in *C. japonica* cells (Kato et al., in preparation). Transient RNAi is also useful for analyzing the transcriptional network. In fact, the application of RNAi in berberine biosynthesis has suggested that primary metabolism in tyrosine biosynthesis and secondary metabolism from tyrosine to berberine are independently controlled.

Our results also suggested that this efficient transient RNAi system might be used to evaluate gene function when combined with adequate systems for evaluating gene function. While our initial attempts to detect the corresponding changes in the metabolite profile by HPLC/LC-MS failed, modification of the experimental system to minimize the residual alkaloid interference detected by HPLC/LC-MS has made it possible to detect the RNAi effect on the norcoclaurine synthase gene to shut down berberine biosynthesis (Fukusaki et al., in preparation). We believe that transient RNAi using in vitro-synthesized dsRNA is a very practical and efficient method for evaluating the functions of genes such as transcriptional factors as well as the effects of metabolic engineering in plant protoplasts.

Development of differential RNAi (dRNAi), comprehensive RNAi for gene families, and a novel gene-substitution method based on dRNAi for functional genomics

The transient RNAi system is effective for analyzing gene function at a cellular level, but the characterization of gene function in whole plants requires a much longer time span. While some transient RNAi systems in lower plants have been reported to be transmitted to the next generation (Kawai-Toyooka et al. 2004), the effectiveness of transient RNAi usually lasts about a week (An et al. 2003, 2005; Dubouzet et al. 2005). One possible application of transient RNAi in whole plants is to use a transitive effect of RNAi, and it has been discussed whether or not transitive RNAi works for endogenous genes in plants (Vaistij et al. 2002). Transitive RNAi is the spread of RNA targeting caused by secondary siRNA, which is generated from dsRNA that extends upstream of the primary target region by RNA-dependent RNA polymerase in *C. elegans* and plants (Figure 1). Therefore, we first examined this critical question of whether isogene-selective RNAi is possible in a gene family, which has highly conserved sequences, since many plant proteins are encoded by redundant genes that constitute multigene families in plants.

Polyploidy has played a prominent role in the evolution of plant species. Although gene redundancy may have advantages in nature, it often makes polyploids poor choices for genetic analyses. Some redundant genes

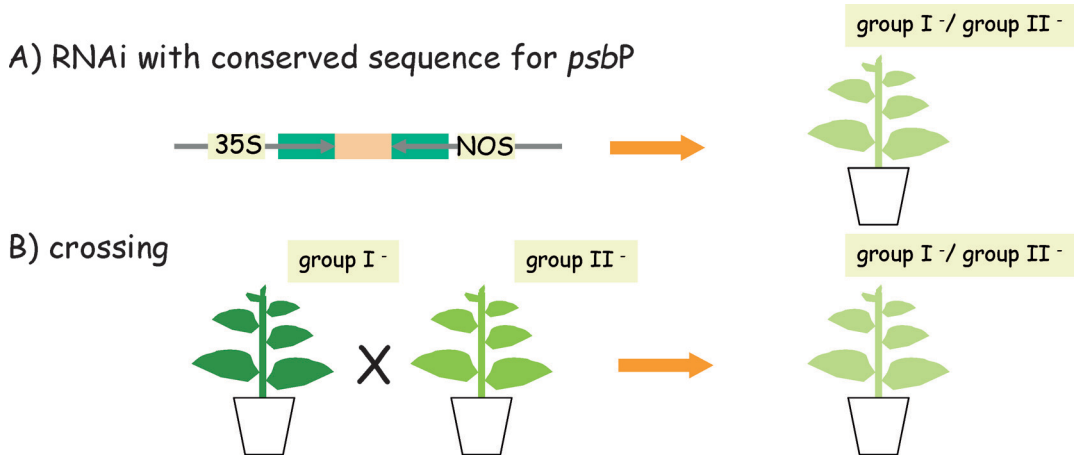

Figure 3. Comprehensive RNAi. A) Comprehensive RNAi with a conserved sequence in the gene family. B) Comprehensive RNAi can be induced by crossing of independent RNAi target transformants. Successful pyramiding of the silencing phenotype indicates that the RNAi machinery is not a limiting factor for a certain level and crossing is a practical way to obtain comprehensive RNAi transformants.

are expressed at a similar time and place, and produce proteins with an equivalent function. On the other hand, other redundant genes are expressed in specific tissues or under different conditions, and might produce proteins with different functions. Thus, a method that can be used to modify the expression of a desired set of genes would be useful for investigating the functions of genes in a multigene family. The RNA interference (RNAi) technique is an effective strategy for circumventing problems associated with gene redundancy. In RNAi, careful selection of a unique or conserved region of the target sequences ensures that a specific member or multiple members of a gene family can be silenced. In fact, we have shown that RNAi can down-regulate specific members by using a sequence-specific region such as the 3′-untranslated region (UTR) as a trigger sequence (Ifuku et al. 2003; Fukusaki et al. 2004; Miki et al. 2005). Furthermore, RNAi facilitates the generation of dominant loss-of-function mutations in polyploid plants, even with short conserved dsRNA, such as that 37 nucleotides long (Ifuku et al. in press; Yamamoto et al. 2005).

As shown above, RNAi offers several advantages for the study of functional genomics in plants. The primary advantage is its ability to specifically target the chosen gene. Since RNAi is a homology-dependent process, careful selection of a unique region of the target sequence can ensure that a specific member of a gene family is silenced, or multiple members of a gene family can be silenced by RNAi for highly conserved sequences and the combination of individual specific RNAi transformants (Figure 3A, B). In this way, redundancy is not a limitation. Furthermore, variable levels of gene silencing can be achieved in different transgenic lines using the same RNAi construct (Wesley et al. 2001), which allows for the selection of lines with various degrees of silencing.

In one of our experiments, Sato's group silenced a *psb*P gene family in amphidiploid *Nicotiana tabacum* with RNAi. PsbP protein is an extrinsic 23-kDa protein in the oxygen-evolving complex (OEC) of photosystem II (PSII) (Seidler 1996; Ifuku et al. 2004). Although PsbP is considered an interesting protein in PSII evolution, the physiological function of PsbP has rarely been reported in higher plants, partially because of gene redundancy. We have used a method called "differential RNAi (dRNAi)" and subsequent complementation/substitution in dRNAi to establish transgenic tobacco plants with various amounts and compositions of PsbP members. The *psb*P isogenes were differentially silenced by RNAi using the 3′-untranslated region of the *psb*Ps as a silencing trigger (Figure 4A) (Ifuku et al. 2003). Next, the extra *psb*P genes without the 3′-untranslated region were complementarily transformed into the above silenced plants, in which an introduced exogenous psbP was accumulated while differential silencing of the endogenous target was maintained (Figure 4B, C) (Ishihara et al. in press). The term "dRNAi" was first used by Laatsch et al. (2004), who showed that endogenous gene expression could be suppressed selectively without affecting the expression of a co-introduced recombinant version of the same protein in human cells. Our independent success with dRNAi in plants suggests that this technique may be widely applicable to experiments that complement the knock-down phenotype of RNAi-silenced plants or replace the function of endogenous protein with exogenous (recombinant or mutated) protein.

Successful dRNAi has indicated that transitive RNAi secondarily produced by RdRp with siRNA reported for *C. elegans* scarcely functioned in the case of endogenous genes, such as tobacco PsbP (Ifuku et al. 2003), *Torenia* chalcone synthase (Fukusaki et al. 2004), and rice Rac genes (Miki et al. 2005). A similar difference in the

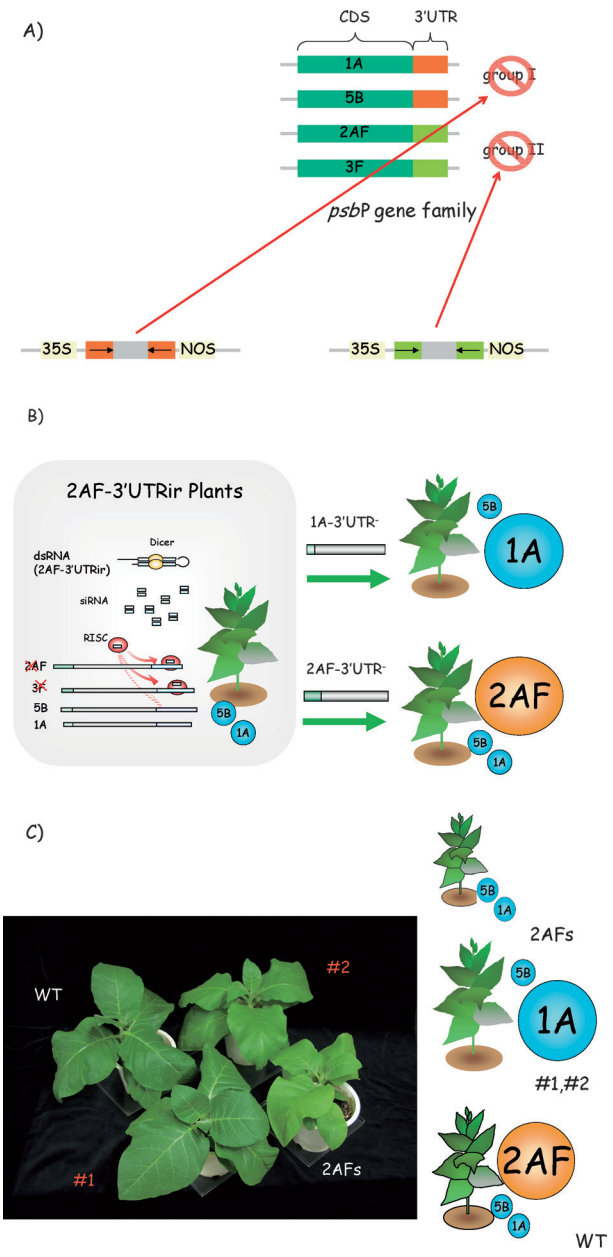

Figure 4. Development of differential RNAi (dRNAi) and a novel gene-substitution method based on dRNAi. In amphidiploid *N. tabacum*, PsbP is nuclear-encoded by a multigene family of four isogenes, which can be divided into two groups according to their sequence similarity (group–I: 1A and 5B; group–II: 2AF and 3F). Their nucleotide sequence similarity in the coding region is very high; those of the 5″- and 3′-UTRs of these clones are close to 90% within each group, and below 50% between groups (Ifuku et al. 2003). A) Isogene-specific RNAi, "differential RNAi (dRNAi)", was shown to be possible by using an RNAi construct with an inverted repeat of unique 3′UTR. B) Complementation in dRNAi. When the *psb*P isogenes were differentially silenced by RNAi using the 3′-UTR of the *psb*Ps, extra *psb*P genes without the 3′-UTR were complementarily transformed into the above silenced plants to modify the composition of PsbP isoform. C) Introduced exogenous *Psb*P was accumulated while differential silencing of the endogenous target was maintained.

recognition of endogeneous and exogeneous genes in RNAi was also found for the systemic transfer of RNAi. While the RNAi effect was transmitted from stock to scion in the case of GFP (Figure 5A) (Himber et al. 2003), such systemic transmission of RNAi was not observed for small subunits of ribulose bisphosphate carboxylase/oxygenase or PsbP genes (Figure 5B) (Himber et al. 2003; Ifuku et al. in preparation). This discrimination of endogenous and exogeneous genes was clearly not due to the lack of multiple gene silencing, since comprehensive RNAi can be achieved with a conserved sequence or with the crossing of individual RNAi transformants. While we have not yet characterized the detailed mechanism, a similar mechanism is also seen for TGS with DNA methylation (Miki and Shimamoto 2005).

RNAi and future application

Development of efficient RNAi vectors for rice functional genomics

Since the rice genome was recently sequenced (International Rice Genome Sequencing Project 2005), the functional identification of rice genes has become increasingly important. While various tagged lines have been generated, not enough tagged genes are available for the extensive study of gene function. To help identify the functions of genes in rice, Ko Shimamoto's group at the Nara Institute of Science and Technology has developed a Gateway vector, pANDA, for RNA interference of rice genes (Miki and Shimamoto 2004). This vector can be used for *Agrobacterium*-mediated transformation of rice and allows for the easy and fast construction of efficient RNAi vectors. In the construct, hairpin RNA derived from a given gene is transcribed from a strong maize ubiquitin promoter, and an intron is placed on the 5′ end upstream of inverted repeats to enhance RNA expression. This vector suppressed mRNA expression in more than 90% of the transgenic plants examined, indicating that RNAi clearly functions in monocot cells. A similar vector, pANDA-mini, was also developed for direct transfer into leaf cells or protoplasts. pANDA vector was also successfully used for the characterization of a flowering-time control gene and disease-resistance genes in rice (Hayama et al. 2003; Lieberherr et al. 2005).

While tissue-specific and quantitatively regulated RNAi has not yet been characterized in detail, various RNAi vectors have been developed. Intron containing self-complementary RNAi vector has been reported as the most efficient RNAi vector, and it is possible that the spacer intron acts as a transcription enhancer (Wesley et al. 2001). Using the *Wx* gene, the key gene in amylose synthesis which is specifically expressed in pollen and endosperm (Itoh et al. 2003), Kimiko Itoh's group at

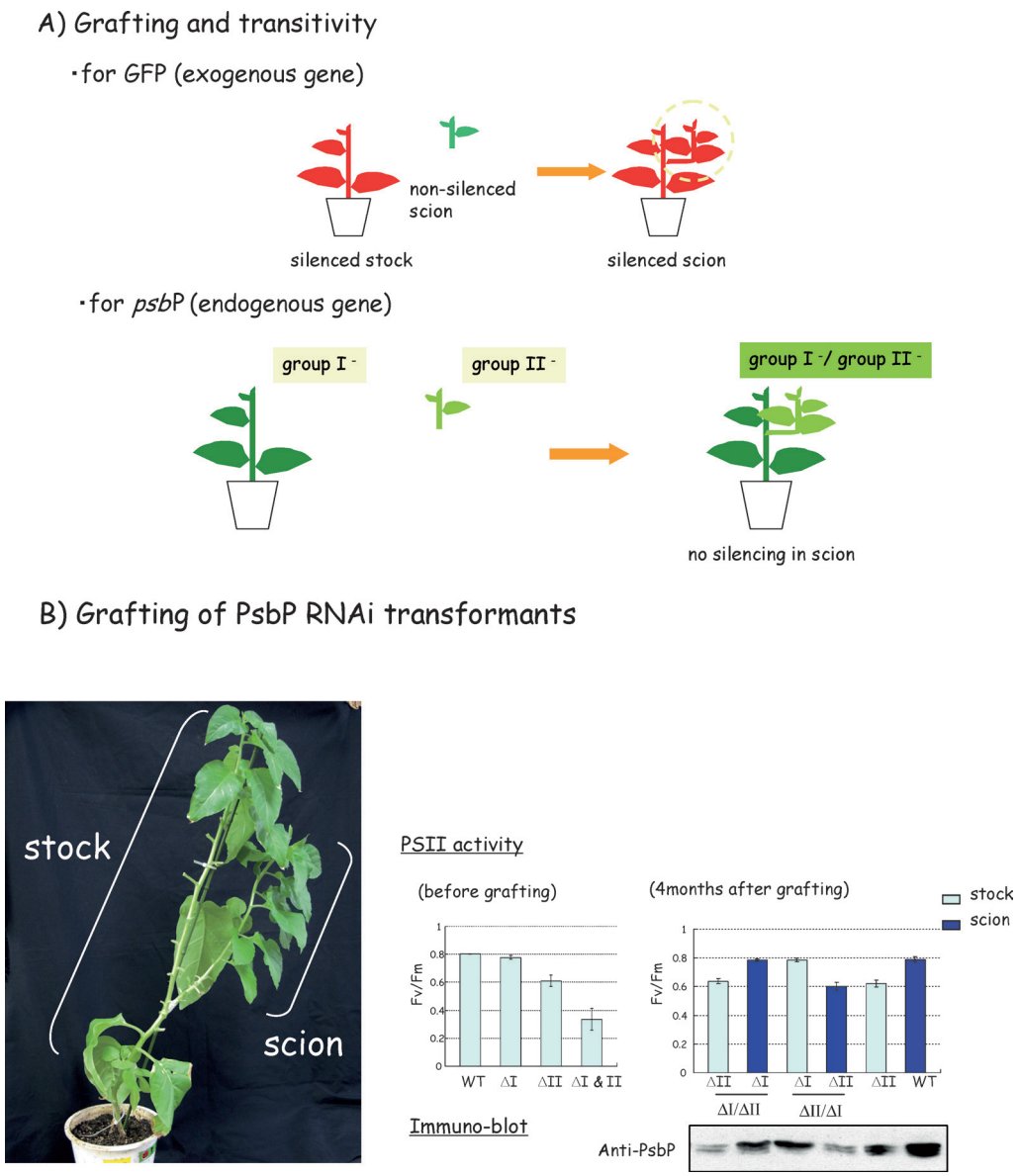

Figure 5. RNAi and transitivity. A) Grafting experiments showed that RNAi silencing was not transmitted systemically in the case of an endogenous gene (e.g. *psb*P), whereas a foreign gene such as GFP was shown to be subjected to systemic silencing. B) Grafting experiments with *psb*P RNAi transformants. ΔI, ΔII indicate transgenic tobacco transformed with RNAi vectors for group I or group II of *psb*P isogene. ΔI&II, ΔI/ΔII and ΔII/ΔI are Comprehensive RNAi transformants for both groups grafted tobacco produced with ΔI as scion and ΔII as stock, and vice versa (Ifuku et al., unpublished data).

Niigata University investigated the endosperm-specific disruption of gene function and quantitatively regulated RNAi (see also Minireview by Itoh et al. in this issue). They constructed endosperm-specific ihp-dsRNA vectors targeted to 5′UTR *Wx* mRNA, pWRI-A and pWRI-B. pWRI-B carried a single mutation at the 5′ splice site of the 1st intron and this mutation reduced the mature transcript of the dsRNA, whereas pWRI-A carried the wild type. These vectors were transformed into Wx^b rice, and the resulting transgenic plants *WRI-A*/Wx^b and *WRI-B*/Wx^b were tested for spatial and quantitative control of silencing efficiency (Tanno et al. 2005). Both vectors efficiently disrupted *Wx* mRNA function among 96–100 % of the transgenic rice plants tested and the degree of *Wx* gene suppression in *WRI-B*/Wx^b endosperms was weaker than that in *WRI-A*/Wx^b endosperms . Their results also showed that the *Wx* promoter regulates endosperm-specific RNAi and controlling the splicing efficiency can quantitatively regulate the suppressive effect on RNAi.

RNAi and metabolic engineering

Higher plants produce a wide variety of secondary metabolites, including more than 25,000 terpenoids, about 8,000 phenolic compounds, and about 12,000

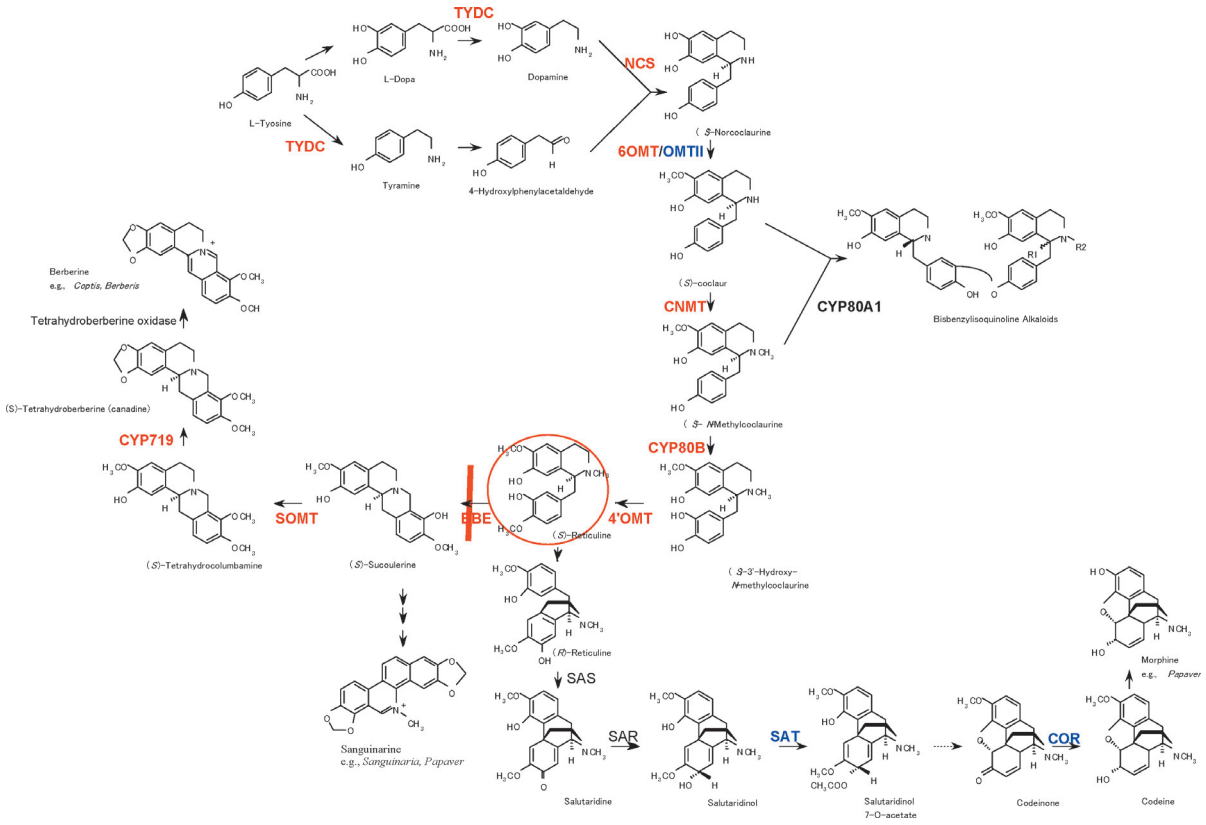

Figure 6. Metabolic engineering by RNAi. The isoquinoline alkaloid biosynthetic pathway and the target of metabolic engineering by RNAi.

alkaloids (Croteau et al. 2000). These chemicals are used as dyes and fragrances, and in important pharmaceuticals. While several strategies have been proposed to increase the production of secondary metabolites in plant cells, the identification of biosynthetic genes is the main obstacle in the molecular engineering of secondary metabolism (Sato and Yamada, in press). Furthermore, the diversity of this metabolism hinders the industrial production of a desired chemical due to contamination by structurally related compounds.

Isoquinoline alkaloid biosynthesis is one of the most well-characterized pathways in the secondary metabolism of plant cells and many biosynthetic genes have been isolated (Hashimoto and Yamada 2003; Shitan et al. 2003). Thus, isoquinoline alkaloid biosynthesis is one of the most suitable models for metabolic engineering. *Coptis japonica* scoulerine-*O*-methyltransferase has been introduced into California poppy cells to create a new branch in the metabolic pathway and to produce novel compounds (Sato et al. 2001). As the next step in molecular engineering, Sato's group examined the efficiency of pathway-trimming, since the complexity of metabolites lowers the production of target molecule in the pathway as well as the difficulty of purification. In isoquinoline alkaloid biosynthesis, (*S*)-reticuline is a branch-point intermediate that is involved in the biosynthesis of many types of isoquinoline alkaloids; morphine, codeine, papaverine, berberine and sanguinarine all originate from this compound. Furthermore, berberine bridge enzyme cDNA has been isolated from California poppy cells (Hauschild et al. 1998), which makes it possible with metabolic engineering to shut-off the pathway to accumulate reticuline (Figure 6). However, a previous attempt to accumulate reticuline with the antisense RNA-mediated suppression of berberine bridge enzyme (BBE) in California poppy was unsuccessful, although this pathway was blocked and biosynthesis of the end-product sanguinarine was considerably reduced (Park et al. 2002, 2003).

Sato's group examined the power of RNA interference (RNAi) technology to completely down-regulate the target enzyme reaction to accumulate the intermediate. Transgenic California poppy cells transformed with BBE RNAi vector (BBEir cells) showed a marked reduction of BBE expression and accumulation of the pathway intermediate reticuline (Fujii et al. in preparation). This is the first successful report of target-gene silencing to produce an important precursor in secondary metabolism. On the other hand, Allen et al. (2004) reported reticuline accumulation in transgenic opium poppy with codeine reductase RNAi vector, although reticuline is not a precursor of codeine reductase. The off-target effect of RNAi and/or complicated metabolic

feedback effects on metabolite accumulation are also important questions in metabolic engineering. Interestingly, transgenic California poppy BBEir cells also produced a methylated derivative of reticuline, laudanine, which could scarcely be detected in control cells, suggesting that the accumulation of substrate could be the switch that activates a substrate-derived reaction.

RNAi and perspectives

RNAi and developmental gene regulation

While this review mainly summarizes recent results regarding RNAi through PTGS, plant cells also silence the gene through DNA-methylation-mediated TGS at the transcriptional level. It has been shown that small RNAs, via RNA interference, direct the modification of proteins and DNA in heterochromatic repeats and transposable elements (Lippman and Martienssen 2004; Herr et al. 2005). DNA methylation is a key epigenetic determinant that controls parent of origin-specific gene expression (imprinting) in mammals, where methylation is erased and re-established in each generation. In contrast, the epigenetic states of gene expression in flowering plants are often inherited unchanged over many generations. To understand the role of DNA methylation in plant development, Tetsuji Kakutani's group first examined the expression of the late flowering mutant gene FWA during normal development. They found that wild-type *FWA* exhibits imprinted (maternal origin-specific) expression in endosperm. Since endosperm does not contribute to the next generation, the activated *FWA* gene need not be silenced again (Kinoshita et al. 2004). Furthermore, they characterized the low DNA-methylation mutant *ddm1* in *Arabidopsis* and revealed that several endogenous genes as well as transposons can be de-suppressed by the reduction of DNA-methylation (Kakutani et al. 2004). Further analysis in rice also indicated the existence of a novel system of gene regulation/recognition in plant cells.

RNAi and virus suppressors

In plants, RNAi is an immune system directed against viruses (Hamilton and Baulcombe 1999; Mallory et al. 2001; Kasschau et al. 2003; Baulcombe 2004). During RNA virus infection, long dsRNAs derived from the replication intermediates of viral RNAs trigger RNAi and degrade viral RNA. To escape these RNAi-mediated defenses, viruses have a counter-defense strategy against RNAi. Over 20 RNAi suppressors have been identified in plants. These suppressors are commonly involved in the enhancement of viral pathogenicity and do not have any obvious sequence similarity. RNAi suppressors have been suggested to act at different steps in the RNAi pathway. Kazuyuki Mise's group characterized several RNAi suppressors. First, the NSs protein of Tomato spotted wilt virus has been characterized as the first RNAi suppressor in negative-strand RNA viruses, and it has been shown that NSs could interfere with multiple steps in the RNAi pathway. Furthermore, they showed a novel mechanism of RNAi suppression by Red clover necrotic mosaic virus (RCNMV), which requires multiple viral components, including viral RNAs and putative RNA replicase proteins (Takeda et al. 2005). RCNMV interferes with the accumulation of siRNA in RNAi induced by a hairpin dsRNA and also interferes with microRNA, which is generated from endogenous hairpin RNA precursors and is involved in the regulation of development (Bartel 2004).

As the above summary indicates, the importance of RNAi machinery, including non-coding RNA such as miRNA, has rapidly expanded in functional genomics, including studies in genome networks and system biology (Mallory and Vaucheret 2004). Continued progress in this exciting field is expected.

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