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RNAi Vectors for Manipulation of Gene Expression in Higher Plants

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Abstract: RNA interference (RNAi) is a homology-dependent gene silencing technology in which small interfering RNAs (siRNAs) direct RNA cleavage or DNA methylation. After transcription of an RNAi cassette including inverted repeat sequences against the target gene and a spacer fragment, the resultant transcript forms a hairpin-like structure. The stem region of hairpin RNAs is processed into siRNAs. Here we focus on the structural properties of RNAi vectors that affect the silencing efficiency, and caveats in the evaluation of RNAi phenotype are discussed. Subsequently, several RNAi applications including simultaneous silencing of multiple gene sequences and specific silencing of a member in the gene family were discussed. In addition a newly developed RNAi technology, artificial microRNA, is also introduced.

INTRODUCTION

RNA interference (RNAi) discovered by Fire *et al.* [1] can ectopically reduce the expression of a target gene. RNAi-based techniques have been employed as effective experimental tools for the elucidation of gene function. According to the accumulation of genomic sequence information of various plants species, the analysis of functionally unidentified genes has been dramatically increasing. Such efforts enable identification of the genes conferring valuable traits. Inactivation of undesirable genes has also been reported in the breeding of plants with increased values for nutrition and food processibility. For example, RNAi has been applied to the production of cotton seeds with high-stearic and high-oleic acids [2, 3], soybean producing seed oil with increased stability at high temperatures [4], tomato fruits with increased carotenoid and flavonoid content [5] and high lysine corn for animal food [6]. Since RNA silencing (including RNAi) share a common mechanism with plant defense mechanisms against invasive viruses, RNAi was used for developing plant immunity as soon as the RNAi vector system was available [7]. Flower quality is also the target of RNAi-based breeding. Rapeseeds with increased flowers [8] and roses with blue-coloured petals [9] were produced by using RNAi technology.

RNAi is a conserved mechanism in a wide range of eukaryotic organisms except for *Saccharomyces cerevisiae* [10, 11]. RNAi inactivates gene expression in a sequence-specific manner. Double-stranded RNA (dsRNA) is an effective trigger of RNAi. In mammalian cells, nematodes and flies, RNAi can be induced by direct introduction of dsRNAs. In contrast, RNAi in plants is usually established by transformation with a construct that produce hairpin RNAs. Chuang and Meyerowitz [12] first demonstrated that efficient RNA silencing in *Arabidopsis thaliana* can be conferred by an RNAi construct. They showed that introduction of an RNAi vector resulted in the silencing of a homeobox gene equivalent to that of the corresponding null mutant. The plant RNAi vector consists of an inverted repeat harboring target sequences under the control of a strong promoter. The inverted repeat sequences are separated by a spacer fragment. After transcription, inverted repeat sequences form a dsRNA structure (the so-called stem) and a spacer forms the loop of a hairpin RNA, respectively (Fig. **1**). RNase III enzymes called Dicer or Dicer-like protein (DCL) process dsRNAs into 21 ~ 25-nt-long small interfering RNAs (siRNAs) [13-15]. *Arabidopsis* has four DCL genes (*DCL1* ~ *DCL4*). In most RNAi plants, dsRNAs are produced under the control of a strong promoter, and resulting high dsRNA levels bypass the true genetic requirement of RNAi and activate different RNAi-related pathways. Thus, partially redundant DCL activities are apparently involved in the plant RNAi. Indeed, Dunoyer *et al.* [16, 17] showed that *DCL4* is specifically required for 21-nt siRNA production from the moderately expressed transgene. Then siRNAs are incorporated into RNA-induced silencing complex (RISC) which consists of several proteins including the Argonaute (AGO) protein. RISC is presumably located in the P-body, an RNA degradation center in the cytoplasm [18]. In *Arabidopsis*, ten predicted AGO family members (*AGO1* ~ *10*) have been identified. The enzyme responsible for mRNA cleavage in RNAi is called a slicer. AGO1 is the main slicer involved in RNAi, and its binding to siRNAs and mRNA cleavage activity were demonstrated *in vitr*o [19]. After RISC-mediated mRNA cleavage, the resulting degraded products are further subjected to the exonucleolytic degradation [20, 21].

Mette *et al.* [22] showed that transcription of inverted repeat sequences against the nopaline synthase promoter caused cytosine methylation of the corresponding promoter region in the genome. This RNA-directed DNA methylation (RdDM) is followed by the abolishment of transcriptional activity of the methylated promoter. Requirement of a histone deacetylase in RdDM suggests that RdDM is associated with heterochromatinization of the targeted promoter region [23, 24]. In fact, siRNAs against the centromere region are essential for formation and maintenance of the heterochromatin state of the centromere [25]. The pattern of dimethylation at H3K9, a heterochromatic maker, also changed in the rice RdDM [26]. Condensation of chromatin blocks the interaction of *trans*-acting factors with a *cis*-acting element in

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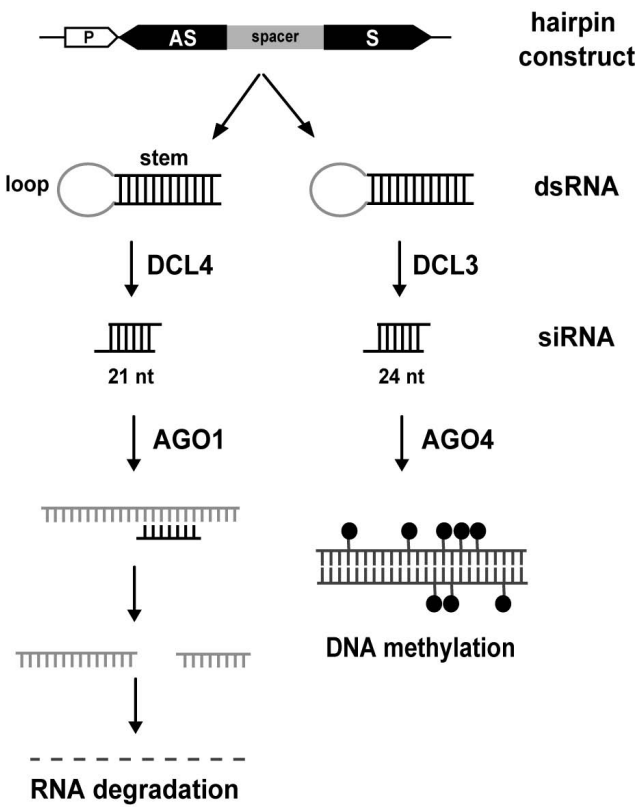

Fig. (1). Plant silencing pathways induced by an RNAi vector.

An RNAi construct produces long dsRNAs after transcription. Two distinct DCL enzymes process this dsRNA. DCL3 processes dsRNAs into 24-nt-long siRNAs which direct cytosine methylation on the homologous genomic loci. DCL4 probably produces 21-nt-long siRNAs. The siRNA incorporated into AGO1 guides the endonucleolytic cleavage of homologous RNA, and siRNAs at AGO4 guide the methylation of cytosine in the complementary genomic sequences. P, promoter; AS, antisense oriented target sequence; S, sense-oriented target sequence.

the promoter. More than 20 *Arabidopsis* genes, such as AGO4, DCL3, etc., are considered to be involved in RdDM [27]. The sequence specificity for cytosine methylation is likely determined by a specific class of 24-nt long siRNAs [25, 28].

Taken together, RNAi can potentially repress the expression of target genes by two different pathways; one is *via* mRNA cleavage and the other is *via* transcriptional inactivation. In plants, both silencing pathways can be controlled by the introduction of RNAi vectors. In this review, we address technical aspects of RNAi vector-mediated control of gene silencing in plants; namely the structural properties of RNAi vectors required for efficient silencing and several applications with RNAi vectors in plants. Readers should refer to several other reviews for the detailed mechanisms of RNAi [15, 29, 30].

DESIGNING RNAI VECTORS FOR EFFICIENT SILENCING

Choice of Target Region

The inverted repeat region against the target gene forms a dsRNA structure which serves as the substrate of Dicer. Thus the sequences and length of the stem region directly affect RNAi efficiency. Which part of a target gene should be chosen for acquisition of maximum silencing effect? Wesley *et al.* [31] constructed inverted repeat sequences against two different regions of a cDNA encoding an *Arabidopsis* flowering repression protein. One construct contained an entire cDNA sequence and another harbored the 3′ two-thirds of the cDNA. Almost all transformants with these two RNAi cassettes flowered earlier than the wild-type plants. In contrast, Heilersig *et al.* [32] showed that the 3′ sequences were a less efficient silencing inducer than the sequences from the 5′ and middle region of the rice starch synthase gene, *GBSSI*. There are few reports examining the relationship between the location of target region and resulting RNAi efficiency. At least to our knowledge, there are no genes harboring the region resistant to RNAi. As mentioned later, selection of the target region would be an important factor determining the specificity of RNAi.

RNAi efficiency has been investigated in terms of the length of the stem region. In animal cells, long dsRNAs induce the interferon response that eventually leads to cell death [33]. To avoid this cellular response, short hairpin RNA molecules with a 25~30-nt long stem have been used. Direct introduction of dsRNA into cells is effective for induction of RNAi in higher plants [34]. However, in most cases of plant RNAi, hairpin constructs that produce transcripts with a long stem region (about 100~1,000-nt in length) have been used. These RNAi vectors are premised on the insertion of cDNA fragments that are easy to handle (Table **1**). The bottom and upper threshold of the cDNA length in the effective size range is about 100 nt and 1,000 nt, respectively. Heilersig *et al.* [32] investigated RNAi efficiency in potato plants transformed with several RNAi vectors harboring inverted repeats with different sizes (488~1,300-nt in length). A stem region longer than 1,100 nt was less effective in silencing. The longest length of stem region showing successful silencing is 1,492 nt of the 5′ chalcone synthase (*CHS*) gene in petunias [35]. The most frequently chosen length of the stem region is about 200~500 nt, and these hairpin constructs showed effective silencing in various plant species including monocotyledonous ones [36].

Choice of Spacer Sequence

The spacer sequence allows us to construct an RNAi vector more easily, since replication of inverted repeat sequences without a spacer is unstable in *Escherichia coli*. Spacer sequences have been prepared from the β-glucuronidase (*GUS*) gene [12], green fluorescence protein (*GFP*) gene [37], and several introns [31, 38-41]. The enhanced RNAi effect can be often observed by using an intron spacer rather than the *GUS* or *GFP* spacers [31, 38, 42]. One possible reason for the enhancement of intron-mediated silencing is the splice-activation effect. The self-annealing of sense and antisense region of the transcript would be promoted after removing the intronic spacer sequences, and the resulting dsRNA molecules would be effectively transported into cytoplasm *via* nucleocytoplasmic transport receptors [43]. Smith *et al.* [38] demonstrated that almost 100% of plants transformed with an intron-containing hairpin construct showed silencing. This report had a very strong impression on many researchers, following which RNAi cassettes were frequently constructed by using an intron spacer.

Table 1. Stem Length of the RNAi Vector and Silencing Efficiency

Target Gene	Host Plant	Stem Length (nt)	Silencing Efficiency[a]	Reference
Δ12 fatty acid desaturase	Cotton	98	+++	Wesley *et al*. [31]
		853	++	
Flowering repression gene (FLC1)	Arabidopsis	400	+++	Wesley *et al*. [31]
		650	+++	
Starch synthase (GBSSI)	Potato	488, 519, 548[b], 618	+++	Heilersig *et al*. [32]
		504, 548[b], 1100	+	
		1300	-	
Nicotine N-demethylase	Tobacco	99	+	Gavilano *et al*. [41]
		298	+++	
Luciferase	Tobacco	300	+++	Akashi *et al*. [122]
		500	+++	

[a]+++, strong; ++, intermediate; +, weak; -, none.
[b]these two stems harbor the sequences with same length but they correspond to distinct region of the target gene.

Several intronic sequences, not all, have been shown to enhance the expression of the cognate genes as a *cis*-acting element [44-47]. The ubiquitin promoter in combination with the intron gave a high expression of downstream genes in rice [48-50]. In fact, an intron of the maize ubiquitin gene was placed 5′ upstream of inverted repeat sequences in order to enhance dsRNA expression in rice [51]. Thus, it is also possible that the intron spacer strengthens the RNAi effects by intron-mediated enhancement in the cognate transcript level and not by the splice-dependent promotion of self-annealing. Hirai *et al.* [52] investigated the effect of intron spacer on RNAi efficiency using a hairy root assay system. The 1st intron of the *NtFAD3* gene encoding a tobacco α-linolenate synthase gave a moderate silencing efficiency, even though the splicing itself was efficient [52]. Similar or less silencing efficiency of RNAi cassettes using an intron spacer has been reported in comparison with cassettes using non-intronic sequences [39, 53, 54]. These results indicate that the use of intronic sequences as a spacer is not always required for the establishment of efficient silencing.

The relationship between spacer length and silencing efficiency was also investigated by using a hairy root assay system [52]. Several *GUS* spacers with different lengths ranging from 100 to 1800 nt were used in the construction of RNAi vectors. Although these RNAi constructs have the same stem region, they conferred different silencing efficiencies. The strongest RNAi effect was obtained in RNAi constructs with 500- and 1,000-nt *GUS* spacers and not a 100-nt one [52]. The RNAi construct against the tomato ACC oxidase gene with a short spacer (7 nt in length) conferred an efficient silencing than that with a 1,002-nt spacer sequence [55]. In contrast, 1,100-bp and 150-bp-long spacers showed nearly the same silencing efficiency of a *GBSSI* gene in potato plants [32]. In conclusion, the spacer sequence certainly influences the silencing efficiency, but we cannot predict which RNAi cassettes can confer strong or weak silencing when different spacers are used. When an RNAi cassette against the *NtFAD3* gene was constructed using a 100-nt *GUS* spacer, the resulting moderate silencing was associated with the accumulation of its hairpin RNA. Thus moderate silencing was attributed to an inefficient processing of its hairpin RNA by Dicer [52]. In most cases, hairpin RNA is rapidly degraded into siRNA and its steady-state level is very low. If different silencing degrees are required, use of several spacers with different lengths or different sequences are one of the meaningful approaches of RNAi.

Promoter Sequences and Other *cis*-Acting Elements

The degree of silencing is apparently proportional to the level of siRNAs, and "strong" promoters have been used in the construction of RNAi vectors as follows, the cauliflower mosaic virus 35S promoter (p35S) [41, 56, 57], soybean lectin promoter [58], *Arabidopsis rbcS* promoter [59, 60], rice ubiquitin promoter [61, 62] and *Chrysanthemum rbcS1* [63]. In addition, the *cis*-acting elements that potentially assist in the transcription of an RNAi cassette have been used. One such element is the intronic sequence (see also previous section) [51]. Another possible *cis*-element is the matrix attachment region (MAR). As was the case of the sense transgene, expression of the RNAi construct should suffer position effects. Transgenes integrated into or near the highly condensed chromatin regions often undergo condensation, resulting in a reduction of transcription. MARs are AT-rich sequences that bind specifically to the nuclear matrix and potentially reduce or eliminate position effects [64-67]. Insertion of a MAR identified from the human interferon β gene into the short hairpin construct enhanced the stability and degree of RNAi in animal cells [68]. Li *et al.* [60] investigated the effect of a MAR derived from the tobacco *rb7* gene on RNAi efficiency and stability. However, this MAR had no discernible influence on silencing in transgenic poplar plants. Since MARs with no effects on the reduction of variability in transgene expression levels have also been reported [69-72], there is room to improve RNAi efficiency if the MARs from different sources are incorporated into RNAi vectors.

siRNA Sequences Generated from the Hairpin RNA

Llave *et al.* [73] determined the siRNA sequences that had been generated from the transiently expressed RNAi cassette against the *GFP* gene. The distribution of cloned siRNA sequences showed that some siRNAs partially shared their sequences with other different siRNA clones. Some clustering of siRNAs along the stem region was observed, indicating that siRNAs are apparently non-randomly distributed. One most likely explanation for this asymmetry in siRNA production is that each arm of a nascent hairpin RNA carries the folded regions. These highly structured regions are recognized as a small hairpin structure, and then will be processed by the plant DCL. Similar asymmetry in siRNA generation has been observed in the plant virus-induced RNA silencing [74]. Another possible explanation is that loading of small RNAs into AGO proteins is specified by the 5' terminal nucleotide. In fact, most 21-nt small RNAs with 5' terminal U were predominantly associated with AGO1 [75]. The asymmetry in the siRNA population may result from poor incorporation of siRNAs with 5' terminal nucleotide other than U, enhancing their turnover.

Assay System for Evaluation of RNAi Efficiency

When RNAi is used in the functional analysis of indispensable genes for life, severe silencing causes a lethal phenotype. Control of the degree of silencing may be required in such a case, and an assay system for the comparison of silencing efficiency among the RNAi vectors would be valuable for this purpose. Hellens *et al.* [76] developed a transient assay for quantification of RNA silencing. In this assay, an RNAi vector is introduced into tobacco leaf cells by agroinfiltration. The inverted repeat sequences against the firefly luciferase (LUC) gene and two independent expression constructs of *LUC* and Renilla luciferase (REN) genes are included in a single T-DNA. The degree of silencing can be assessed in a few days by measuring the decrease of chemiluminescence of LUC relative to that of REN. This system would be valuable for the analysis of RNAi vector structures themselves, but was not suitable for elucidation of the degree of silencing of specific target gene sequences. Panstruga *et al.* [40] developed an assay that is based on transient expression using two fluorescent proteins as a marker (GFP) for transfected cells and the second (red fluorescent protein; RFP) as translational fusion with the target gene. Co-introduction of this test plasmid with an RNAi vector against the target gene resulted in a specific decrease of RFP fluorescence. This assay allows us to evaluate the degree of silencing of the target gene, and would be valuable for the design of RNAi vectors that confer a severe and mild trigger of RNAi. These methods can be used as an easy and rapid tool to analyze the effectiveness of RNAi without long-term plant-transformation experiment. If the phenotype of a target gene can be determined using root cells, the assay system using hairy roots provides the strict evaluation of the effects of RNAi constructs on target gene expression in a relatively short experimental term [52, 77].

GENERATION OF IMPROVED RNAI VECTORS

High-Throughput RNAi Vectors

Construction of RNAi vectors is a time-consuming and laborious work since two fragments corresponding to the antisense and sense region of the stem should be subcloned into a binary vector. To inactivate multiple gene functions, a rapid and easy method for construction of RNAi vectors is certainly helpful. High-throughput plant RNAi vectors have been constructed to fulfill such a demand. For instance, single step construction of an RNAi vector has been developed. pHELLSGATE [78] and pANDA [51] vectors allow the assembly of an inverted repeat structure by Gateway recombination technology [79-81]. In the construction of these RNAi vectors, PCR products for the target gene are generated with primers flanking attB1 and attB2 sites, and then recombined into two cloning sites with attP1 and attP2 sequences by BP clonase. Although this single step reaction was easy to construct, the resulting long recombination-related sequences weakened the silencing efficiency [82]. Therefore, a two-step recombination procedure, which leaves short sequences on the resulting RNAi vector, has been recommended for efficient silencing [81]. Several pHELL SGATE-related RNAi vectors have been developed for RNAi in monocotyledonous plants and for inducible RNAi [79].

For conventional cloning, pHANNIBAL [31], pKANNIBAL [83], pSAT [84] and pSH [52] are available. In these RNAi vectors, PCR fragments of the target gene are produced by using primers with restriction sites, and cloned successively into both upstream and downstream region of the spacer to become the two arms of the hairpin construct.

Inducible RNAi Vectors

RNAi technique cannot be applied to genes whose silencing interferes with plant regeneration or causes embryo lethality or severe pleiotropic phenotypes. For example, RNAi against the *Arabidopsis* Zinc finger transcription factor gene, *ZAT6*, was lethal [85]. Complete depletion of γ-tubulin by RNAi was lethal at the cotyledon stage [86]. To counter this problem, the lethal and sterile phenotypes are eluded by using an inducible RNAi vector which can confer transient and local silencing. Ethanol or estrogens are used as chemical regulators of these inducible promoters. In the case of an ethanol-inducible vector, a transcriptional regulator, AlcR, is constitutively expressed and the RNAi cassette is inserted behind the *alcA* promoter. After ethanol treatment, AlcR binds to the *alcA* promoter and transcription of the downstream RNAi sequences is activated [87].

Another inducible RNAi vector is developed by the combination of Cre/*loxP*-mediated recombination and a chemical inducing system, in which the stringent control of expression of an RNAi cassette can be achieved. A chimeric transcriptional factor, XVE, is constitutively expressed. When XVE binds to estrogens, the activated XVE induces the transcription of a Cre recombinase gene. The resulting Cre recombinase removes a fragment that blocks transcription of an RNAi cassette [88].

The pHELLSGATE vector-based inducible RNAi vector is also available [89, 90]. This system uses a modified RNAi cassette from pHELLSGATE under the control of the pOp6 promoter. A synthetic transcription factor, LhGR, is constitutively expressed by p35S. In the presense of dexamethasone, LhGR binds to pOp6 and the downstream RNAi cassette is transcribed. These inducible RNAi vector systems allow the rapid induction of RNAi across the whole plant or in limited tissues under investigation [91].

amiRNA

A new RNAi vector was recently developed in which the structure of hairpin RNA mimics that of microRNAs (miRNAs). This new RNAi vector is called as artificial microRNAs (amiRNAs) [92]. The precursor RNA of miRNAs is transcribed from the genomic *bona fide* loci distinct from the locus encoding target RNA [93, 94]. miRNA precursors show a characteristic feature: mismatches or bulges are included in the stem. One miRNA-miRNA* duplex (* means a dispensable RNA strand in miRNA-mediated silencing) is produced after processing by DCL. In the amiRNA vector, the target sequence was inserted as miRNA sequences and the complementary sequence was designed as miRNA* [92, 95]. The position and number of mismatches and bulges in amiRNA closely resemble a representative miRNA. The single small RNA produced from an amiRNA has a few mismatches against the target RNA. The 3′ terminal base of resulting small RNAs is not homologous to target mRNA by avoiding the nucleotide extension by an RNA-dependent RNA polymerase [92].

For designing amiRNAs, WMD (Web MicroRNA Designer) platform based on a set of provided criteria is available [96]. Qu *et al.* [97] demonstrated that an amiRNA against the 2b gene of Cucumber mosaic virus (CMV) efficiently conferred resistance to CMV infection on a tobacco plant. Since the amiRNA vector produces single small RNA species as a guide of RISC, the selection of a sequence corresponding to the miRNA region is extremely important as was the case of the construction of short hairpin RNAs in animal cells. If potential targets are properly predicted in the genome-wide range, non-specific silencing by amiRNAs is markedly reduced in comparison with the silencing *via* orthodox RNAi vectors. In addition, high specificity by amiRNAs can be adapted for allelic-specific inactivation. In this respect, amiRNA technology may be suitable for model plants such as *Arabidopsis* and rice. The *Arabidopsis* amiRNA library provided by Drs. Hanon, Martienssen, McCombie and their colleagues is now released on the market, in which each 22,000 gene is targeted by 3 different amiRNAs. These amiRNAs resemble the miR319a structure and they are transcribed under the control of p35S. The amiRNA vector is constructed by using a binary vector, and is ready to transform plants [98]. *Arabidopsis* amiRNAs would open up an easy way of specific inactivation of target genes.

FUNCTIONAL KNOCKDOWN OF SINGLE GENE OR MULTIPLE GENE SEQUENCES

Since sequence specificity is determined by hybridization of siRNAs to corresponding target mRNAs, siRNAs consisting of sequences shared by several genes cause simultaneous silencing of multiple gene sequences (Fig. **2a**). Efficient silencing of several members of a gene family is demonstrated in the families for rice *OsRac* genes [61], *Solanum americanum PIN2* [57], and the soybean ω-3 fatty acid desaturase [4]. A single RNAi construct against the conserved region in topspoviruses can interfere with the infection of four different topspoviruses in tomato [99]. The nucleotide similarity that allows simultaneous silencing is important to estimate the effects of RNAi against the gene family member. In this respect, Xu *et al.* [100] showed that simultaneous silencing would occur between genes showing sequence identity with at least 22-nt continuous stretches.

In contrast, in order to analyze the function of specific member of a gene family without breaking down the expression of others, use of the 3′ untranslated region (UTR) is effective (Fig. **2b**). Specific silencing against single member of tobacco *psbP* [101], torenia *CHS* [102], the *Arabidopsis PRF* [103] and rice *OsRac* [61] gene families was achieved by using 3′ UTR sequences as a trigger of silencing. In addition, specific inactivation of a target gene can be carried out by using the amiRNA vector in *Arabidopsis* (Fig. **2c**, see previous section).

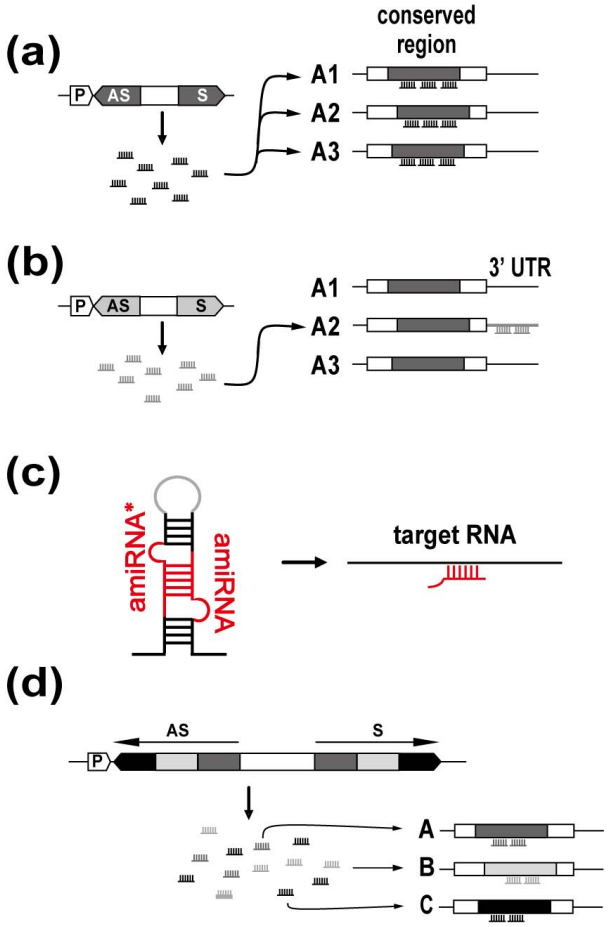

Fig. (2). mRNA Targeting by different strategies.

(**a**) When the conserved regions among the related gene sequences (referred to as A1 ~ A3) is used for construction of an RNAi vector, siRNAs generated from the resultant RNAi vector can simultaneously inactivate the multiple target genes. (**b**) When the 3′ untranslated region (UTR) is used to generate an inverted repeat sequence, this RNAi vector can suppress the expression of a specific member of related genes. (**c**) The amiRNA vector produces a transcript that mimics the miRNA precursor. The miRNA and miRNA* are replaced with target gene sequences and corresponding complementary sequences, respectively. In the amiRNA vector, single small RNA guide the RISC. (**d**) To inactivate multiple target genes (each of them have unrelated sequences from the others; referred to as A, B and C), a chimeric arm of the stem consisting of the target genes can be used. After processing, the siRNAs corresponding to the targeted genes are simultaneously produced at once.

If the expression of two or more genes were to be suppressed, successive transformation would be required. However, there are only a limited number of selectable marker genes that are available for practical use. For simultaneous RNAi against several unrelated targets, Zhong *et al.* [104] constructed an RNAi vector in which two RNAi expression cassettes are tandemly arranged in single T-DNA. Similarly, conjugation of several RNAi cassettes onto a single binary plasmid was reported [84]. When these multiple RNAi cassettes were driven by the same promoter, the resulting transformants potentially have the risk of attenuating the RNAi due to the cross silencing in the same promoters. These problems may be overcome by incorporation of multiple target sequences into single RNAi cassette. Miki *et al.* [61] developed an RNAi construct harboring multiple trigger sequences in a single inverted repeat structure. They showed simultaneous successful silencing of double or triple target genes by single transformation (Fig. **2d**). This method can save the promoter and selectable marker gene sequences.

RNAi VECTOR FOR INDUCTION OF RDDM

The structural properties of promoter hairpin RNA expression vectors have not been investigated well. p35S is silenced by promoter-targeted RNAi in petunia [35] and rice [26]. In petunia plants, promoter hairpin cassettes carrying a whole region of p35S and a 261-bp enhancer region showed efficient reduction of transcriptional activity, but an RNAi construct against the 123-bp minimal promoter for p35S showed limited repression of transcriptional activity [35]. We investigated the region conferring an efficient transcriptional silencing of p35S in tobacco plants. These constructs contained inverted repeats corresponding to four distinct regions of p35S (Fig. **3**). Leaves of the transgenic tobacco which express the *GUS* gene under the control of p35S were re-transformed with Ri plasmid and these RNAi constructs. These four distinct RNAi vector reduced the GUS activity to similar levels, indicating that transcriptional activity of p35S can be repressed by using RNAi cassettes harboring about 200-bp-long promoter sequences (Fig. **3**).

It is important for most of researchers to assess whether or not the siRNA can inactivate the promoters of endogenous genes. In this respect, only sporadic evidence has shown its validity. Inactivation of endogenous promoters by RdDM has been shown in petunia [35], *Arabidopsis* [105], maize [106], and potato [107]. In contrast, six of seven endogenous rice genes were resistant to RdDM-mediated promoter inactivation although *de novo* methylation itself occurred in these promoter sequences [26]. Since RdDM-associated transcriptional silencing is apparently influenced by the local surrounding genomic condition of the target promoter locus [108], reduction of promoter activity may significantly vary among the endogenous promoters of interest, ranging from no reduction to an almost complete knockdown level by the effects of the surrounding genomic sequences.

OFF-TARGET EFFECT

Recent investigations have demonstrated that transcripts having less than 100% complementarity with an siRNA can be cleaved by the RNAi pathway [109-111]. The down-regulation of mRNAs other than the intended target in RNAi is referred to as off-targeting, which deteriorates the specificity and applicability of RNAi [112]. In fact, a computational

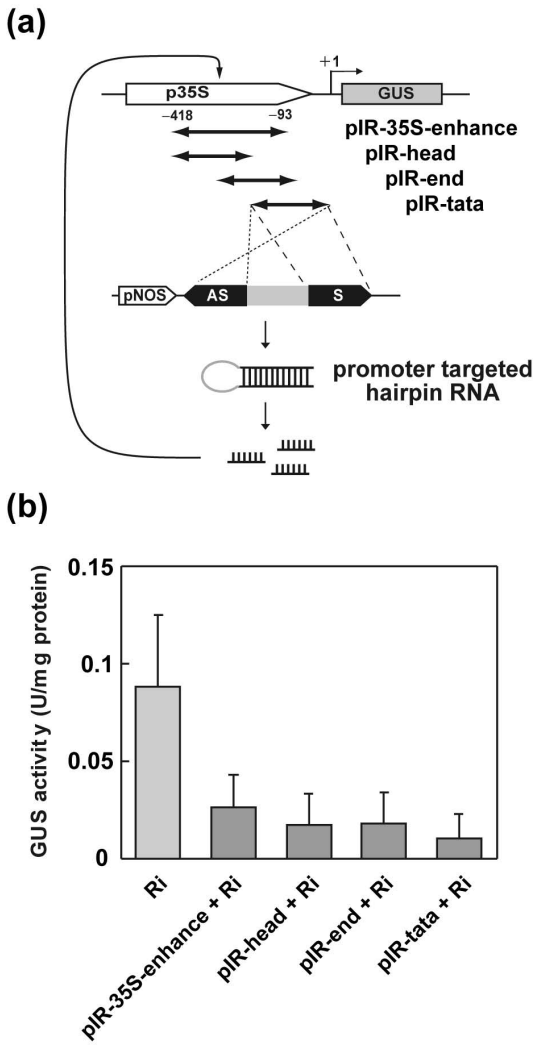

Fig. (3). RdDM-mediated inactivation of p35S.

The RNAi constructs against four different regions of p35S (**a**) and their RNAi phenotype (**b**). The inverted repeat sequence was transcribed by a nopaline synthase promoter (pNOS). The RNAi cassette is inserted into pSMAH621 (personally distributed by Dr. Ichikawa). Each RNAi vector contains an inverted repeat corresponding to the enhancer region (-418 ~ -93 from the transcriptional start point, pIR-35S-enhance). 5′ region (-418 ~ -219, pIR-head), central region (-293 ~ -94, pIR-end), or 3′ most region containing the TATA box (-200 ~ +1, pIR-tata). These RNAi vectors and an Ri plasmid were introduced into transgenic tobacco leaves expressing the GUS gene under the control of p35S (a representative line, G36). The hairy roots were induced on Murashige-Skoog medium supplemented with 3% sucrose, 0.3% Gellan gum, 750 mg/L Augmentin (GlaxoSmithKline) and 10 mg/L hygromycin B. The GUS activity of each hairy root was determined using an AURORA GUS kit (ICN Biomedicals) with a LUMAT LB9506 luminometer (Berthold) by counting relative chemiluminescence units. The GUS enzyme unit was calculated by using Ampullaria β-glucuronidase (Wako Pure Chemical Industries) as a standard. Vertical lines indicate SD (n = 10). These RNAi constructs reduced the GUS activity by about 68% ~ 88% when compared with those of control hairy roots.

study using the genome and transcriptome sequence data of human, worm and yeast suggested that the risk of off-target

effects by siRNAs cannot be ignored [111, 113]. The Jackson group found a way out of this problem to reduce unintended down-regulation in animal cells, which includes chemical modification in siRNAs that prevented the incorporation of sense siRNA into RISC, reducing siRNA concentration, and applying more rigorous bioinformatics to siRNA design [114, 115]. As mentioned in a previous section, the off-target effect by amiRNA is potentially reduced when compared with that of a traditional RNAi vector. In contrast, preparation of experimental evidence of the off-target silencing by a traditional RNAi vector is quite difficult because of the complexity of siRNA species generated from hairpin RNAs with a long stem. In fact, different phenotypes, which may be due to the off-target effect, are observed between the null mutant and RNAi transformants [116]. To confirm that an RNAi phenotype is due to the "on-target" silencing, Thomas *et al.* [117] investigated the phenotypic variation among plants that had been transformed with two or more RNAi vectors against different regions of a single target mRNA. Kumar *et al.* [118] investigated whether or not the RNAi phenotype can be complemented by a synthetic gene that encodes the same protein as the native gene but uses different nucleotide sequences. If the phenotype of interest is due to on-target silencing, it will be completely reversed by expression of the synthetic gene. For RNAi to become a more reliable technology in plants, specificity and functionality of RNAi must be properly addressed.

Another potential factor of the off-target effect is the spreading of RNAi target by the amplification of siRNAs. This amplification process (termed transitivity) results from the action of RNA-dependent RNA polymerase [119]. Although transitivity may increase the incidence of off-target inactivation, it rarely occurs against endogenous genes [61, 120, 121]. This characteristic feature alleviates the off-target effects in RNAi analyses against endogenous genes.

CONCLUSION

The development of RNAi technology confers an efficient knockdown of gene expression and it has replaced antisense technology or cosuppression that show less efficient silencing, namely low silencing frequency or weak depletion of target mRNA. Although it seems paradoxical, the RNAi vector systems that allow modulating the degree of silencing, namely weak or strong silencing, would be helpful for further fine investigation of gene function. In addition, an easy and simple method for validation of on-target silencing has been desired. Although there are a lot of subjects to be solved for popularization in wide-range plant species, the development of an amiRNA vector would provide a more sophisticated RNAi system. The rapid progress of RNAi technology may enable to control the expression of a number of genes at a once, and to regulate target mRNA at a level that corresponds to researchers' demands in the near future.

ABBREVIATIONS

AGO = Argonaute

amiRNA = artificial microRNA

CHS = chalcone synthase

CMV = Cucumber mosaic virus

DCL = Dicer like

dsRNA = double-stranded RNA

GFP = green fluorescence protein

GUS = β-glucuronidase

LUC = firefly luciferase

MAR = matrix attachment region

p35S = Cauliflower mosaic virus 35S promoter

RdDM = RNA-directed DNA methylation

REN = Renilla luciferase

RFP = red fluorescence protein

RISC = RNA-induced silencing complex

RNAi = RNA interference

siRNA = small interfering RNA

UTR = untranslated region

WMD = Web microRNA designer

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