Chapter

RNAi Induced Gene Silencing Journey from Simple dsRNA to High-Throughput Intron Hairpin RNA Construct in Crop Improvement

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Abstract

RNA interference (RNAi) is the process in which short interfering RNA (siRNA) act to inactivate the expression of target genes. The tremendous work done by many research groups around the globe have contributed in deciphering the RNAi pathway. Understanding the role of siRNA and machinery involved in RNAi pathway led to application of this pathway as technique in therapeutic applications and crop improvement. The specificity of siRNA in interacting the target sequence helped to understand the complex pathways and role of major genes involved. Here we have reviewed the journey involved in understanding RNAi pathway and in vitro use of dsRNA to induce RNAi machinery against the target gene. It explains the advances achieved in vector construction from simple RNA construct to high-throughput ihpRNA constructs for higher efficiency in target-specific gene silencing for crop improvement.

Keywords: RNAi, crop improvement, vector construction, ihpRNA

1. Introduction

The introduction of recombinant DNA techniques and the progression in gene transfer technique and plant regeneration strategies in the 1980s empowered the trading of genes not only between plants of various species but also from viruses, bacteria, fungi, and animals into plants. This capacity to transform plants with transgenes gave unparalleled chances for crop improvement and also widened the scope for upgrading of strategies in genetic engineering. One such strategy developed was RNAi for gene silencing.

The RNAi pathway was first observed in the nematode in response to double-stranded RNA (dsRNA) which resulted in sequence-specific gene silencing. It was then developed as weapon to fight an ongoing war between viruses and living organism from decades. Researchers have been constantly working toward combating virus invasion especially in crops. In this context in 1986, the American plant virologist Roger Beachy and his partners demonstrated an experiment against the tobacco mosaic virus (TMV) infection in tobacco plants. Using gene transformation technique,

they introduced a transgene derived from the coat protein gene of tobacco mosaic infection in tobacco plants. This virus-based transgene acted against the TMV and protected plant from infection [1]. This finding inspired significant research movement around the globe and prompted numerous reports of protection against a range of viruses infecting plants. However, the underlying mechanism of resistance against viruses using pathogen-derived gene at the molecular level was not known at that time.

The study of Lindbo and Dougherty [2] gave an insight to the happenings at the molecular level. They show that transforming plants with transgenes that can express RNAs of viral origin, but which could not be translated into proteins, could initiate a mechanism that could degrade both the mRNA from the introduced transgene and the genomic RNA of the inoculated virus and lead to repression of the infection by the virus. They also found great similarities with a phenomenon called posttranscriptional gene silencing (PTGS)—which had been described using transgenes of nonviral origin.

Transgene-induced posttranscriptional gene silencing has been detected in plants [3] where it is called co-suppression and in fungi where it is called quelling [4]. In animals, PTGS can be induced by dsRNA in a process called RNA interference (RNAi) [5]. In plants, PTGS can also be induced by viruses expressing host genes in a process called virus-induced gene silencing (VIGS) [6]. Viruses themselves can be the targets of the PTGS machinery [6]. It was proposed that if a cell expresses molecules of the same RNA sequence at a level higher than a certain threshold, this induces a mechanism that specifically destroys that RNA [7]. This proposition led to changes in the way of construct designing and preparation, and the journey from dsRNA construct to high-throughput ihpRNA construct began.

2. Double-stranded RNA construct for gene silencing

Based on the Lindbo and Dougherty proposition [2], constructs were made with an aim to increase the copy number of virus-derived transgenes [8]. Constructs contained two sense copies or two antisense copies or contained both one sense and one antisense copy. The transgenic plants with these different constructs were analyzed for virus resistance. They observed that tobacco plants transformed with construct containing both sense and antisense copies showed higher protection against PVY virus. Based on the analysis, they concluded that the production of dsRNA was causing the silencing effect and not the number of gene copies.

Hence, the scientist thought of introducing dsRNA of the target RNA in plant to trigger the silencing mechanism. An experiment was conducted to induce immunity against potato virus Y (PVY) in tobacco plant [8]. Tobacco plants were transformed with gene constructs that encoded the Pro sequence in the sense or antisense orientation or in both orientations and were challenged with PVY. Less than 15% of the Pro[s] or Pro[a] lines showed resistance to PVY, whereas the lines containing both sense and antisense genes showed 44–54% of resistance to PVY. This result suggests that the sense and antisense mRNA in the same cell elicit the PVY resistance. Similar reports were obtained in rice cells transformed with a GUS-derived hairpin (hp) gene. The cells with the GUS gene alone gave a strong blue color, but those with both the GUS gene and the GUS-hp gene remained white [9].

3. Hairpin RNA construct

Further the construct was improved with a transgene expressing RNA that folds back and hybridizes with itself to form a structure like a hairpin, instead of making

two separate RNAs which hybridize into dsRNA [8–11]. With ongoing research and use of RNAi technique for gene silencing, it was observed that silencing efficiency of RNAi vectors can be increased if the factors like selection of target sequences, the inverted repeats, size of the repeats, vectors, use of spacers or introns in the RNAi cassette, promoters, etc. are efficiently worked upon in vector construction. Studies on these parameters were conducted for improvement of hpRNA construct to increase silencing efficiency.

4. Improvement of hpRNA construct

4.1 Selection of target sequence

Overall efficiency of RNAi is dependent on RNA-protein interactions during siRNARISC assembly and activation [12, 13]. Hence, a systematic analysis of 180 siRNAs targeting the mRNA of two genes was done to identify siRNA-specific features that are likely to contribute to efficient processing at each step of RNAi [14]. Based on the analysis, characters like low G/C content, a bias toward low internal stability at the sense strand 3′-terminus, lack of inverted repeats, sense strand base preferences (positions 3, 10, 13, and 19), etc. were found to be affecting siRNA functionality. An algorithm was designed to incorporate all the eight criteria for the selection of potent siRNA for facilitating the functional gene knockdown studies.

A similar study was conducted to decipher the relationship between short interfering RNA (siRNA) sequence and RNA interference in three mammalian and *Drosophila* cells by analyzing 62 targets of 4 exogenous and 2 endogenous genes [15]. Based on analysis certain rules were formulated for designing effective siRNAs capable of inducing highly effective gene silencing in mammalian cells. Rules included the sequence conditions, viz., the presence of A/U at the 5′ end of the antisense strand and G/C at the 5′ end of the sense strand, the presence of at least five A/U residues in one-third of the antisense strand toward 5′ end, and the absence of any GC stretch of more than 9 nt in length. These rules indicated that siRNAs which satisfy all these conditions will increase the gene silencing efficiency in mammalian cells.

The investigation was done on the use of RNA interference for obtaining resistance against Cotton leaf curl Multan virus (CLCuMV) [16]. Three hairpin RNAi constructs were produced containing either complementary-sense genes essential for replication/pathogenicity or noncoding regulatory sequences of CLCuMV. All three RNAi constructs significantly reduced the replication of the virus in inoculated tissues. However, the systemic movement of the virus was controlled by only one of the constructs (CLCRNAiRepTrAPREn/pFGC), possibly because it spanned three virus-encoded genes: the replication-associated protein (Rep), the replication enhancer protein (Ren), and the transcriptional activator protein (TrAP). Also, the ability of virus to infect plant was compromised as the expression of TrAP was downregulated. TrAP is a transcription factor possibly involved in suppression of silencing machinery. Hence, both the target sequence and the levels of identity between the construct and target sequence determined the outcome of RNAi-based resistance against the virus.

On studying the effects of the structure, position, and sequence of a target RNA on RNAi using 47 constructs for inhibition of firefly luciferase activities by siRNAs targeted to TAR motif, it was observed that the efficacy of siRNAs depended mainly on the target sequence [17]. Statistical analysis of the data collected on the sequence preferences indicated that some nucleotides at specific positions are positively or negatively correlated with the efficiencies of siRNAs; for example, the siRNAs with an A residue at the 19th nucleotide position from the 5′ end of the sense strand showed relatively high suppressive activities, and siRNAs with a G residue at the

19th nucleotide in the sense strand tended to be less effective. Similar preference (A19 in siRNA; U1 in the miRNA) was observed for miRNA sequences [18]. This preference suggests the importance of the low internal stability of the 5′ terminus of the antisense strand and a possible functional contribution of a U at the 5′ end to the activities of both siRNA and miRNA [12, 13]. A significant negative correlation was also observed between the GC content of the 3′ half of siRNAs (in particular, from the 12th to the 19th nucleotide) and the activities of siRNAs. These tendencies can also be seen in another report [19].

The first commercialized transgenic papaya carrying the PRSV CP gene was introduced to Hawaii in 1998 to save the remains of the papaya industry [20]. However, transgenic papaya cultivars showed varying levels of resistance against PRSV isolates from other geographical regions. For example, isolates from the Bahamas, Florida, and Mexico have delayed, mild symptoms. Isolates from Brazil and Thailand also have delayed symptoms, but the virus eventually overcomes their resistance. The CP hemizygous line, "Rainbow," is also susceptible to PRSV isolates from Taiwan [21]. Resistance levels therefore were found to be dependent on the variability among CP genes of the isolates [22–24]. The high levels of genetic divergence in PRSV isolates from Hainan caused the failure of transgenic papaya lines that targets specific viral CP gene [25]. CP-transgenic resistance of papaya is expressed in a nucleotide-sequence-homology-dependent manner [26, 27].

The proper selection of a target sequence for a given gene of interest remains one of the most critical components of successful gene knockdown regardless of the RNAi methodology.

4.2 Size of repeats

The silencing efficiency is reported to be proportional to the size of target sequence, and the terminal regions (5′ and 3′) were found to be unaffected by silencing mechanism [28]. This description was based on the experiment conducted to identify the target regions and relative efficiencies of various target regions for silencing of gn1 (β-1,3 glucanase) gene in transgenic tobacco line T17.

Similarly, the effect of length of inverted repeats in tobacco BY-2 cells was tested by co-transformation of a luciferase gene construct and a luciferase dsRNA expression plasmid [29]. The dsRNA expression plasmids targeted to the firefly luciferase gene were constructed with 500- and 300-bp inverted repeats. However, no significant difference in silencing efficiency was observed, and the presence of 300-bp dsRNA was found to be sufficient to suppress the luciferase activity in cultured plant cells. A longer dsRNA did not show any enhancement in RNAi effect.

The less silencing frequency is reported when shorter fragments were used [30]. It was based on constructs with fragments of range 50 bp–1 kb targeted to silence two *Arabidopsis* genes, FLC (flowering locus C) and PDS (phytoene desaturase), successfully [30]. The use of fragments between 300 and 600 bp was recommended to achieve effective silencing.

Differences in silencing efficiency were also observed in *Neurospora crassa* upon introduction of varied size of inverted repeat constructs targeting the albino-1 gene [31]. Higher silencing frequencies were obtained when the length of the repeat for the target albino-1 gene in *Neurospora crassa* was kept above 200 and below 900 nucleotides. A substantial decrease in the silencing efficiency was observed when the repeat size was reduced below 200 nucleotides.

The effect of size of granule-bound starch synthase (GBSSI) sequence in inverted repeat constructs was evaluated, and it was found that, for GBSSI, the small inverted repeat constructs were more efficient silencing inducers than the large inverted repeat constructs. The small inverted repeat constructs with a repeat

size of 500–600 bp and a spacer of about 150 bp were observed to be more efficient silencing inducers than the large inverted repeat constructs where the size of the repeat was 1.1 or 1.3 kb whilst the size of spacer was 1.3 or 1.1 kb [32].

Together, these reports suggest that 300–500 bp is the optimal size for inverted repeat for effective silencing mechanism.

4.3 Vectors

Construction of RNAi vectors takes considerable time and is a tedious task since it involves laborious conventional cloning technology that relies on restriction digestion and ligation of two fragments corresponding to the antisense and sense region of the stem and subcloning into a binary vector. Hence, there is demand for high-throughput plant RNAi vectors for a rapid and easy construction.

For instance, technique involving a single step for construction of an RNAi vector has been developed that facilitates fast and reliable DNA cloning. It is called as gateway cloning technique which is also available with compatibility for *Agrobacterium* sp. binary vector system. The backbone of all Gateway-compatible destination vectors is derived from the pCambia series of binary vectors for *Agrobacterium* sp.-mediated plant transformation. The Gateway recombination site for introduction of a DNA fragment of interest is placed toward the right border of the T-DNA in the pCambia vectors. Most of the T-DNA destination vectors described contain the hygromycin phosphotransferase plant-selectable marker gene. This selectable marker was chosen so that these vectors would be compatible with a large number of insertion lines that are kanamycin-resistant.

For the construction of RNAi vectors using Gateway recombination technology, the PCR products of the target gene are generated with primers flanking attB1 and attB2 sites for recombination with two cloning sites with attP1 and attP2 sequences using BP clonase. pHELLSGATE [30] and pANDA [30] are the vectors that allow the assembly of an inverted repeat structure by Gateway recombination technology [33]. Several pHELLSGATE-related RNAi vectors have been developed for RNAi in monocotyledonous plants and for inducible RNAi [34, 35]. For conventional cloning, pHANNIBAL [36], pKANNIBAL [37], pSAT [38], and pSH [39] 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 regions of the spacer to become the two arms of the hairpin construct. Simultaneously work was done to enhance the efficiency in cloning inverted repeats for RNAi. An all-purpose vector, pGEMWIZ, for assembling the repeat for any *Drosophila* gene was developed [40]. The inverted repeats in pGEM-WIZ are stable in common *E. coli* strains and a fast selection method to correctly identify such clones with repeats is available.

For a simple and efficient construction of intron-containing hpRNA (ihpRNA) vectors, a novel restriction-ligation approach was developed. The system was designed based on the type IIs restriction enzyme BsaI and plant RNAi vector pRNAi-GG based on the Golden Gate (GG) cloning [41]. It required only a single PCR product of the gene of interest flanked with BsaI recognition sequence which can then be cloned into pRNAi-GG at both sense and antisense orientations simultaneously to form ihpRNA construct. The ihpRNA construction process could be completed in one tube with one restriction-ligation step.

4.4 Use of spacers or introns

The silencing efficiency of RNAi vectors was reportedly enhanced by the addition of an intron interposed between the inverted flanking target sequences in

vector. The spacer fragment was observed to give the stability to the inverted repeat sequences. It did not play any role in PTGS and was spliced out during pre-mRNA processing. The process of intron excision from the construct by the spliceosome might help to align the complementary arms of the hairpin in an environment favoring RNA hybridization and promoting the formation of a duplex. Also, splicing may contribute to increase the amount of hairpin RNA by preventing the hairpin's passage from the nucleus or by creating a smaller, less nuclease-sensitive loop [10].

Smith et al. [10] showed that transgene constructs encoding a splicable intron within a hairpin RNA structure can induce PTGS with almost 100% efficiency. The percentage of PVY-resistant tobacco plants obtained by targeting the nuclear inclusion a (NIa) protease gene of PVY with different constructs was 7% for the sense gene, 4% for the antisense gene, 58% for the hpRNA with a nonspliceable loop separating the sense and antisense arms, and 96% for the same hpRNA with a spliceable intron. The high efficiency of intron hpRNA constructs for inducing PTGS and generating virus-immune transgenic plants was confirmed for Cucumber mosaic virus (CMV) [42, 43].

The effect of spacer sequences on silencing potential of RNAi constructs was demonstrated [44]. It was tested by an in vivo assay of the α-linolenic acid content in hairy roots of tobacco with RNAi vectors against ω-3 fatty acid desaturase (NtFAD3) gene responsible for production of α-linolenic acid of root membrane lipids. The frequency of RNA silencing was observed to be more affected by spacer sequences than by spacer length (100–1800 bp). They concluded that it is possible to change the degree of silencing by replacing spacer sequences. They predicted the reason to be interaction of spacer sequences with stem sequences of the hpRNA which affect the formation of a hairpin structure.

4.5 Promoters

The degree of silencing is apparently proportional to the level of siRNAs, and the following "strong" promoters have been used in the construction of RNAi vectors: the cauliflower mosaic virus 35S promoter (p35S) [45–47], soybean lectin promoter [48], *Arabidopsis* rbcS promoter [49, 50], rice ubiquitin promoter [51, 52], and *Chrysanthemum* rbcS1 [53].

RNAi technique cannot be applied to genes whose silencing interferes with plant regeneration or causes embryo lethality or severe pleiotropic phenotypes. In such cases the inducible RNAi vectors are used which can confer transient and local silencing. Ethanol- or estrogen-inducible vectors were developed for transient RNAi expression. 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 [54]. Also, a Cre/loxP-mediated recombination and a chemical inducing RNAi vector were developed for the stringent control of expression of an RNAi cassette. A chimeric transcriptional factor, XVE, was constitutively expressed which when binds to estrogen induces the transcription of a Cre recombinase gene. The resulting Cre recombinase removes a fragment that blocks transcription of an RNAi cassette [55]. Similarly, for controlled expression, the pOp6 promoter was used which could allow the rapid induction of RNAi across the whole plant or in limited tissues under investigation. The induction was controlled by the expression of a synthetic transcription factor, LhGR, which can bind to pOp6 in the presence of dexamethasone and initiate the transcription of downstream RNAi cassette [56]. The pHELLSGATE vector-based inducible RNAi vector is also available [57].

5. Gene silencing by intron-containing hairpin RNA construct

Improvement of hpRNA construct for efficient silencing led to the establishment of ihpRNA construct. Using hpRNA constructs containing sense/antisense arms ranging from 300 to 500 nt gave efficient silencing in a wide range of plant species, and inclusion of an intron in these constructs had a consistently enhancing effect. An intron-spliced hpRNA construct gives a higher proportion of silenced transformants than intron-free hpRNA constructs. The process of intron-splicing aligns the arms of the hpRNA, facilitating their duplex formation in the spliceosome complex, whereas the arms of hpRNAs have to find their self-complementarity by random tethered collisions. Intron-spliced hpRNA facilitates the more efficient and steady levels of duplex RNA formation that are sufficient for PTGS. Similarly, the tighter loop of ihpRNA gives more nuclease-stable and higher steady-state duplex RNA levels than the larger-looped hpRNA [36]. In plants, intron-containing hairpin RNA constructs with a spliceable intron as spacer sequence had the highest efficiency, with 80% to approximately 100% transformants showing silencing of target genes [10, 33, 36].

6. Conclusion

RNA interference has been used to develop efficient strategies to silence targeted genes in a wide range of species. Posttranscriptional silencing of plant genes using antisense or co-suppression constructs usually results in only a modest proportion of silenced individuals. Recent work has demonstrated the potential of the constructs encoding intron-containing self-complementary "hairpin" RNA to efficiently silence genes. The degree of silencing with these constructs was much greater than that obtained using either co-suppression or antisense constructs. Currently, the ihpRNA technology has become one of the most powerful tools for gene discovery and gene engineering in plants.

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