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Chapter 24

# Gene Duplication and RNA Silencing in Soybean

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## 1. Introduction

Soybean, *Glycine max* (L.) Merr., is considered to be a typical paleopolyploid species with a complex genome [1-3]. Approximately 70 to 80% of angiosperm species have undergone polyploidization at some point in their evolutionary history, which is a well-known mechanism of gene duplication in plants [4]. The soybean genome actually possesses a high level of duplicate sequences, and furthermore, possesses homoeologous duplicated regions, which are scattered across different linkage groups [5-8]. Based on the genetic distances estimated by synonymous substitution measurements for the pairs of duplicated transcripts from expressed sequence tag (EST) collections of soybean and *Medicago truncatula*, Schlueter et al. estimated that soybean probably underwent two major genome duplication events: one that took place 15 million years ago (MYA) and another 44 MYA [9].

Gene duplication is a major source of evolutionary novelties and can occur through duplication of individual genes, chromosomal segments, or entire genomes (polyploidization). Under the classic model of duplicate gene evolution, one of the duplicated genes is free to accumulate mutations, which results in either the inactivation of transcription and/or a function (pseudogenization or nonfunctionalization) or the gain of a new function (neofunctionalization) as long as another copy retains the requisite physiological functions [10; and references therein]. However, empirical data suggest that a much greater proportion of gene duplicates is preserved than predicted by the classic model [11].

Recent advances in genome study have led to the formulation of several evolutionary models: a model proposed by Hughes [12] suggests that gene sharing, whereby a single gene encodes a protein with two distinct functions, precedes the evolution of two functionally distinct proteins; the duplication–degeneration–complementation model suggests that duplicate genes acquire debilitating yet complementary mutations that alter one or more sub-

functions of the single gene progenitor, an evolutionary consequence for duplicated loci referred to as subfunctionalization [4, 11, 13]. In addition to this notion, models involving epigenetic silencing of duplicate genes [14] or purifying selection for gene balance [15, 16] have also been proposed. In soybean, differential patterns of expression have often been detected between homoeologous genes [17, 18], which indicates that subfunctionalization has occurred in these genes.

When the extent of subfunctionalization is limited, mutations in only one of multiple cognate gene copies do not often result in phenotypic changes. Therefore, methods that allow suppression of all copies of the duplicated gene are required for analyzing gene function or engineering novel traits. RNA silencing refers collectively to diverse RNA-mediated pathways of nucleotide-sequence-specific inhibition of gene expression, either at the posttranscriptional or transcriptional level, which provides a powerful tool to downregulate a gene or a gene family [19, 20]. Suppression of gene expression through RNA silencing is particularly useful for analyzing the function(s) of duplicated genes or engineering novel traits because it allows silencing of multiple cognate genes having nucleotide sequence identity. In fact, to produce soybean lines that have a novel trait, researchers have frequently used RNA silencing induced by a transgene.

In this review, we describe application of RNA silencing to understand the roles of genes or engineering novel traits in soybean. We describe methods to induce simultaneous silencing of duplicated genes and selective silencing of each copy of duplicated genes through RNA silencing. In addition to intentionally induced RNA silencing, we also refer to naturally occurring RNA silencing. Based on our knowledge of RNA silencing in soybean, we propose a hypothesis that plants may have used subfunctionalization of duplicated genes as a means to avoid the occurrence of simultaneous silencing of duplicated genes, which could be deleterious to the organism.

## 2. Mechanisms and diverse pathways of RNA silencing

Gene silencing is one of the regulatory mechanisms of gene expression in eukaryotes, which refers to diverse RNA-guided sequence-specific inhibition of gene expression, either at the posttranscriptional or transcriptional level [19, 20]. Post-transcriptional gene silencing (PTGS) was first discovered in transgenic petunia plants whose flower color pattern was changed as a consequence of overexpression of a gene that encodes the key enzyme for anthocyanin biosynthesis in 1990 [21, 22]. Similar phenomena have also been reported for plants transformed with various genes, which include virus resistance of plants that have gene or gene segments derived from the viral genome [23, 24]. Because of these findings, gene silencing is thought to have developed to defend against viruses. Several lines of research in plants indicated that double-stranded RNA (dsRNA) is crucial for RNA degradation [25, 26]. The potency of dsRNA to induce gene silencing was demonstrated in *Caenorhabditis elegans* by injecting dsRNA into cells in 1998 [27], and the phenomenon was termed RNA interference (RNAi).

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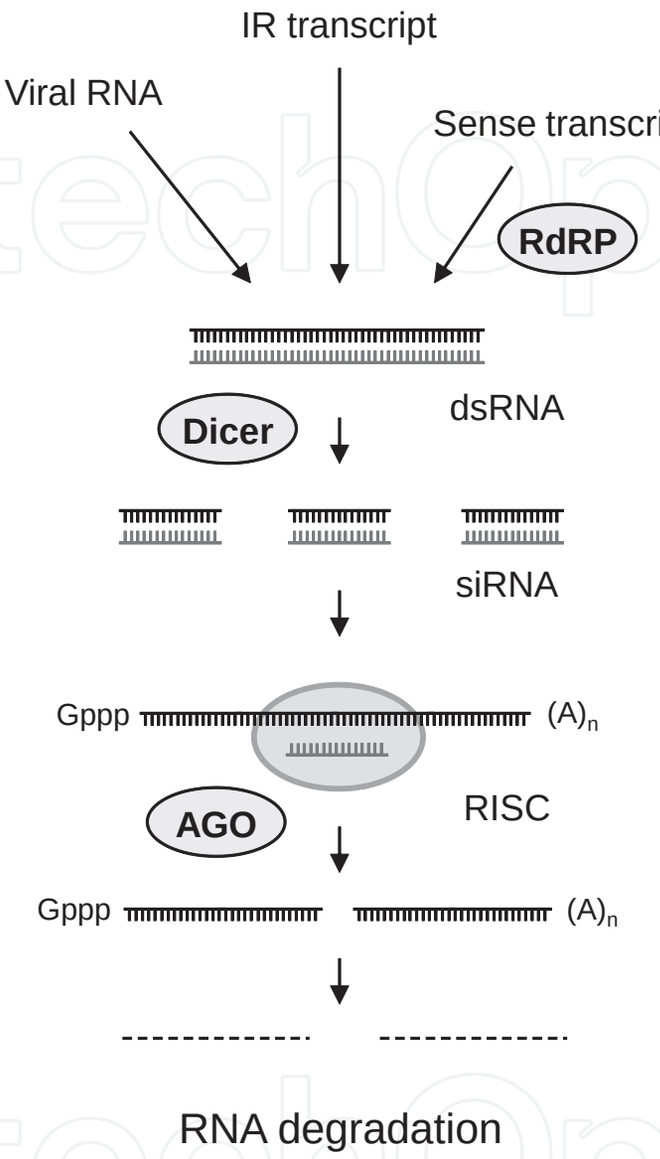


**Figure 1.** Pathways of RNA silencing used to downregulate a target gene through RNA degradation. Posttranscriptional gene silencing is triggered by dsRNA. Transcripts from transgenes that have an IR sequence can form dsRNA. Sense transcripts can produce dsRNA through the synthesis of complementary strand by RdRP. The replication intermediate or duplex structures formed within single-stranded RNA of the viral genome can also provide dsRNA. These dsRNAs are processed into siRNAs by the endonuclease Dicer. The siRNA is loaded into the RISC complex that contains AGO and guides the RISC complex to the mRNA by base-pairing. The RISC complex cuts the mRNA, which is subsequently degraded. Abbreviations: IR, inverted repeat; RdRP, RNA-dependent RNA polymerase; dsRNA, double-stranded RNA; siRNA, short interfering RNA; RISC, RNA-induced silencing complex; AGO, Argonaute.

Subsequent genetic and biochemical analyses in several organisms revealed that PTGS and RNAi share the same pathway and consist of two main processes: (i) processing of dsRNA into 20–26-nt small RNA molecules (short interfering RNA; siRNA) by an enzyme called Dicer that has RNaseIII-like endonuclease activity; (ii) cleavage of RNA guided by siRNA at a complementary nucleotide sequence in the RNA-induced silencing complex (RISC) containing the Argonaute (AGO) protein (Figure 1) [28]. The formation of dsRNA from single-stranded sense RNA was explained by the synthesis of its complementary strand by RNA-dependent RNA polymerase (RdRP). This process provides templates for Dicer cleavage that produces siRNAs and consequently allows amplification of silencing [29]. siRNA is responsible for not only induction of sequence-specific RNA degradation but also epigenetic changes involving DNA methylation and histone modification in the nucleus, which leads to transcriptional gene silencing (TGS) [30]. It has become evident that siRNA plays a role in systemic silencing as a mobile signal [31, 32]. In addition to siRNA, small RNA molecules called micro RNAs (miRNAs) are also involved in negative regulation of gene expression [33]. These gene silencing phenomena that are induced by sequence-specific RNA interaction are collectively called RNA silencing [34, 35].

RNA silencing plays an important role in many biological processes including development, stability of the genome, and defense against invading nucleic acids such as transgenes and viruses [20, 29, 30]. It can also be used as a tool for analyzing specific gene functions and producing new features in organisms including plants [36-38].

## 3. Methods of the induction of RNA silencing in soybean

### 3.1. Transgene-induced RNA silencing

Engineering novel traits through RNA silencing in soybean has been done using transgenes or virus vectors (Figure 1). RNA silencing in some transgenic soybean lines was induced by introducing a transgene that transcribes sense RNA homologous to a gene present in the plant genome, a phenomenon termed co-suppression [21]. This type of silencing was first discovered in transgenic petunia plants that had silencing of *CHS-A* for chalcone synthase [21, 22], in which mRNA transcribed from both *CHS-A* transgene and endogenous *CHS-A* gene was degraded. When sense transcripts from a transgene trigger RNA degradation, the pathway is also referred to as sense (S)-PTGS [19]. To obtain plants that have RNA silencing of a particular gene target, it is possible to generate co-suppressed plant lines as a byproduct of a transformation to overexpress the gene under the control of a strong promoter. However, a more promising method to induce RNA degradation is to transform plants with a construct comprising an inverted repeat (IR) sequence of the target gene, which forms dsRNA upon transcription (IR-PTGS) [39, 40]. This idea was based on the understanding of general mechanisms of RNA silencing in which dsRNA triggers the reaction of RNA degradation. The majority of transgene-induced RNA silencing in soybean have actually been done using such an IR construct. IR-PTGS can also be induced when multiple transgenes are integrated in the same site in the genome in an inverted orientation and fortuitous read-through transcription over the transgenes produces dsRNA.

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An interesting finding reported in soybean is that RNA silencing is induced by a transgene that transcribes inverted repeats of a fatty acid desaturase *FAD2-1A* intron [41]. This result is contrary to the earlier belief that RNA silencing is a cytoplasmic event and intron does not trigger RNA degradation, which has been shown, for example, by using viral vector in plants [42] or by dsRNA injection to *C. elegans* cells [27], although irregular nuclear processing of primary transcripts associated with PTGS/RNAi has been reported previously [43]. The *FAD2-A1* intron-induced RNA silencing led to the understanding that RNA degradation can take place in the nucleus [44]. Although whether RNA degradation in the nucleus is inducible for other genes or in other plants has not been known, this phenomenon is intriguing because the involvement of nuclear events has been assumed for amplification of RNA silencing via transitivity [45] or intron-mediated suppression of RNA silencing [46].

Transcribing a transgene with a strong promoter tends to induce RNA silencing more frequently than that with a weak promoter [47]. For obtaining a higher level of transcription in soybean plants, the *Cauliflower mosaic virus* (CaMV) promoter has been used as in other plant species. Seed-specific promoters, such as those derived from the genes encoding subunits of β-conglycinin, glycinin, or Kunitz trypsin inhibitor, have also been used in soybean to induce seed-specific silencing, one feature that is exploited for metabolic engineering in soybean.

A gene construct that induces RNA silencing has been introduced to the soybean genome using either *Agrobacterium tumefaciens* infection or particle bombardment, which can produce stable transgenic soybean lines that have altered traits. In addition, RNA silencing can be induced in soybean roots using *A. rhizogenes*-mediated transformation, which has been used for gene functional analysis. Methods for soybean transformation have been reviewed elsewhere [48].

### 3.2. Virus-induced gene silencing (VIGS)

RNA silencing has also been induced using a virus vector in soybean. Plants intrinsically have the ability to cope with viruses through the mechanisms of RNA silencing. When plants are infected with an RNA virus, dsRNA of the viral genome is degraded by the infected plants [49, 50]. The dsRNA in the virus-infected cells is thought to be the replication intermediate of the viral RNA [51] or a duplex structure formed within single-stranded viral RNA [52]. The viral genomic RNA can be processed into siRNAs, then targeted by the siRNA/RNase complex. In this scenario, if a nonviral segment is inserted in the viral genome, siRNAs would also be produced from the segment. Therefore, if the insert corresponds to a sequence of the gene encoded in the host plant, infection by the virus results in the production of siRNAs corresponding to the plant gene and subsequently induces loss of function of the gene product (Figure 2). This fact led to the use of a virus vector as a source to induce silencing of a specific gene in the plant genome, which is referred to as virus-induced gene silencing (VIGS) [42, 53, 54]. So far, at least 11 RNA viruses and five DNA viruses were developed as a plant virus vector for gene silencing, as listed previously [37]. Three vectors are now available in soybean: those based on *Bean pod mottle virus* (BPMV) [55], *Cucumber mosaic virus* (CMV) [56], and *Apple latent spherical virus* (ALSV) [57].

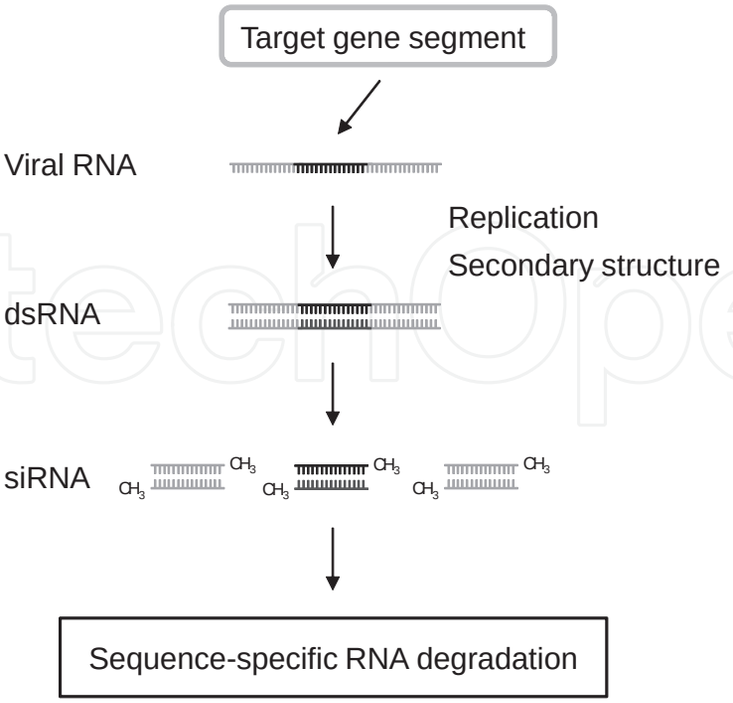


**Figure 2.** Virus-induced silencing of plant endogenous gene. When plants are infected with an RNA virus, dsRNA of the viral genome is degraded by the infected plants. The dsRNA in the virus-infected cells is thought to be the replication intermediate or secondary-structured viral RNA. The viral genomic RNA can be processed into siRNAs. If a plant gene segment is inserted in the viral genome, siRNAs corresponding to the plant gene are produced and subsequently induce sequence-specific RNA degradation of the plant gene.

## 4. Examples of RNA silencing reported in soybean

### 4.1. Metabolic engineering by transgene-induced RNA silencing

To the authors' knowledge, 28 scientific papers that describe metabolic engineering by transgene-induced RNA silencing in soybean have been published up to 2011 [58]. Because soybean seeds are valued economically for food and oil production, most modifications to transgenic soybean plants using RNA silencing are focused on seed components. Metabolic pathways in developing seeds have been targeted in terms of altering nutritional value for human or animals, e.g., changing seed storage protein composition [59, 60], reducing phytic acids [61, 62], saponin [63] or allergens [64], and increasing isoflavone [65]. Metabolic engineering has also targeted oil production [66-72]. These modifications were done by inhibiting a step in a metabolic pathway to decrease a product or by blocking a competing branch pathway to increase a product.

RNA silencing can be induced efficiently in soybean roots using *A. rhizogenes*-mediated root transformation. This method has been used for analyzing roles of gene products in nodule development and/or function, which occurs as a consequence of interaction between legume plants and the nitrogen-fixing symbiotic bacterium *Bradyrhizobium japonicum* [73-78]. The

hairy root system was also used for analyzing roles of a MYB transcription factor in isoflavonoid biosynthesis [79].

Transgene-induced RNA silencing has also been induced in leaf tissues for the β-glucuronidase gene [80] or the senescence-associated receptor-like kinase gene [81] and in calli for the amino aldehyde dehydrogenase gene to induce the biosynthesis of 2-acetyl-1-pyrroline [82].

### 4.2. Disease resistance acquired by transgene-induced RNA silencing

Another focus of modifying soybean plants through RNA silencing is resistance against diseases, particularly to those caused by viruses. Resistance to viruses was achieved by transforming plants with genes or segments of genes derived from viruses and was referred to as pathogen-derived resistance [23, 24, 83, 84]. The resistance did not need protein translated from the transgene [85-87], which led to the understanding that RNA is the factor that conferred resistance to the plants and that the enhanced resistance is acquired via a mechanism analogous to that involved in co-suppression. Using this strategy, soybean plants resistant to *Soybean mosaic virus* (SMV) [88-90], or *Soybean dwarf virus* [91, 92] have been produced.

In addition to resistance against a virus, transgenic soybean plants resistant to cyst nematode (*Heterodera glycines*) have also been produced using RNA silencing [93], in which an inverted repeat of the major sperm protein gene from cyst nematode was transcribed from the transgene. RNA silencing was elicited in cyst nematode after nematode ingestion of dsRNA molecules produced in the soybean plants; as a consequence, reproductive capabilities of the cyst nematode were suppressed. The effects of RNA silencing on controlling *H. glycines* [94] or root-knot nematode (*Meloidogyne incognita*) [95] infection have been assayed in soybean roots using *A. rhizogenes*-mediated transformation. On the other hand, this root transformation method has also been used for analyzing a role of host genes in resistance against diseases caused by *Phytophthora sojae* [96, 97], *Fusarium solani* [98] or cyst nematode [99].

### 4.3. Gene functional analysis by VIGS

An advantage of VIGS is its ease for making a gene construct and introducing nucleic acids to cells. In addition, the effect of silencing can be monitored within a short time after inoculating plants with the virus. Because of these features, VIGS is suitable for gene function analysis [51, 100, 101] and has been used for gene identification via downregulating a candidate gene(s) responsible for a specific phenomenon in soybean. VIGS was used to demonstrate that genes present in the genetically identified loci actually encode the genes responsible for the phenotype: VIGS of the putative *flavonoid 3′-hydroxylase* (*F3′H*) gene resulted in a decrease in the content of quercetin relative to kampferol, which indicated that the putative gene actually encodes the F3′H protein [56]; VIGS of the *GmTFL1b* gene, a soybean orthologue of *Arabidopsis TERMINAL FLOWER1* (*TFL1*) and a candidate gene for the genetically identified locus *Dt1*, induced an early transition from vegetative to reproductive phases, which indicated the identity between *Dt1* and *GmTFL1b* [102]. VIGS has also been used to identify genes involved in resistance of soybean plants against pathogens such as SMV, BPMV, *Pseudomonas syringae* or *Phakopsora pachyrhizi* [103-107].

### 4.4. Naturally occurring RNA silencing

In addition to artificially induced RNA silencing, naturally occurring RNA silencing has also been known in soybean. Naturally occurring RNA silencing, involving mRNA degradation induced as a consequence of certain genetic changes, has been detected based on phenotypic changes. Most commercial varieties of soybean produce yellow seeds due to loss of pigmentation in seed coats, and this phenotype has been shown to be due to PTGS of the *CHS* genes [108, 109]. In cultivated soybean, there are varieties producing seeds with yellow seed coats and those producing seeds with brown or black seed coats in which anthocyanin and proanthocyanidin accumulate. In contrast, wild soybeans (*Glycine soja*), an ancestor of the cultivated soybean, have exclusively produced seeds with pigmented seed coats in thousands of accessions from natural populations in East Asia that we have screened (unpublished data). Thus, the nonpigmented seed coat phenotype was probably generated after domestication of soybean, and humans have maintained the plant lines that have *CHS* RNA silencing. The genetic change that induced *CHS* RNA silencing has been attributed to a structural change in the *CHS* gene cluster, which allows production of inverted repeat *CHS* RNA [110].

The occurrence of RNA silencing that leads to changes in pigmentation of plant tissues has also been reported for the *CHS* genes in maize [111] and petunia [112]. In petunia, a variety 'Red Star' produces flowers having a star-type red and white bicolor pattern, which resembles the flower-color patterns observed in transgenic petunias with co-suppression of the *CHS* genes [113], and in fact, the phenotype was demonstrated to be due to RNA silencing of the *CHS* genes in the white sectors [112]. Breeding of petunia was launched in the 1830s by crossing among wild species. The generation of the star-type petunia plants as a consequence of hybridizations between plant lines suggests that RNA silencing ability can be conferred via shuffling of genomes that are slightly different from each other. These phenomena also resemble the RNA silencing in a seed storage protein gene in rice, which is associated with a structural change in the gene region induced by mutagenesis [114], a case of RNA silencing in nontransgenic plants.

## 5. Diagnosis of an RNA silencing-induced phenotype using viral infection

In the course of the analysis of *CHS* RNA silencing, the function of a virus-encoded protein called suppressor protein of RNA silencing was used to visually demonstrate the occurrence of RNA silencing [108, 111, 112, 115]. These suppressor proteins affect viral accumulation in plants. The ability of the suppressor protein to allow viral accumulation is due to its inhibition of RNA silencing by preventing the incorporation of siRNAs into RISCs or by interfering with RISCs [116]. Because of these features, RNA silencing can be suppressed in plants infected with a virus that carries the suppressor protein. When a soybean plant that has a yellow seed coat is infected with CMV, the seed coat restores pigmentation [108]. This phenomenon is due to the activity of gene silencing suppressor protein called 2b encoded by the CMV. This example typically indicates that, using the function of viral suppressor protein,

we can "diagnose" whether an observed phenotypic change in a plant is caused by RNA silencing. A similar phenomenon has also been detected in maize [111] and petunia [112] lines, both of which have phenotypic changes through naturally occurring RNA silencing of an endogenous *CHS* gene, or a transgenic petunia line that has *CHS* co-suppression [115].

## 6. What do phenotypic changes induced by RNA silencing in soybean indicate?

Soybean is thought to be derived from an ancestral plant(s) with a tetraploid genome, and as a consequence, large portions of the soybean genome are duplicated [7], with nearly 75% of the genes present in multiple copies [117]. In addition, genes in the soybean genome are sometimes duplicated in tandem [118-121]. Our recent studies have indeed shown functional redundancy of duplicated genes in soybean [122, 123]. Such gene duplication can be an obstacle to producing mutants by conventional methods of mutagenesis. In this regard, the gene silencing technique is particularly useful because it allows silencing of multiple cognate genes having nucleotide sequence identity.

Changes in phenotypes as a consequence of inducing RNA silencing have been successful for many genes in soybean as mentioned above. Considering that many genes are duplicated in soybean genome, this fact indicates either that RNA silencing worked on all duplicated genes that have the same function or that the genes were subfunctionalized after duplication, so that RNA silencing of even a single gene of the duplicated genes resulted in the phenotypic changes.

It is of interest to understand whether duplicated genes have identical or diversified functions, which may depend on the time after duplication event and/or the selection pressure on the genes. To analyze the functions of each copy of the duplicated genes, we need to silence a specific copy of the duplicated genes. If the duplicated genes are expressed in different tissues, RNA silencing of both genes can lead to understanding the function of each gene. PTGS by transcribing inverted repeat with a constitutive promoter or VIGS will be suitable for this analysis. An example of such an approach is the VIGS of duplicated *TFL1* orthologues, which are expressed in different tissues. A specific role of one of the *TFL1* orthologues has been identified by VIGS as mentioned earlier [102].

## 7. Methods to induce selective RNA silencing of duplicated genes

When duplicated genes are subfunctionalized with only limited nucleotide changes and are expressed in overlapping tissues, specific silencing of each gene will be necessary for understanding their function(s). Silencing a specific copy of duplicated genes can be achieved by targeting a gene portion whose nucleotide sequence is differentiated between the duplicated genes. A condition that allows this type of silencing involves a lack of silencing of the other copy of duplicated genes even when they have the same sequence in the other portions.

In plants, miRNAs or siRNAs promote production of secondary siRNAs from the 5′ upstream region and/or the 3′ downstream region of the initially targeted region via production of dsRNA by RdRP. These secondary siRNAs can lead to silencing of a secondary target that is not directly targeted by the primary silencing trigger [124]. Studies so far have indicated that such a spread of RNA silencing, called transitive RNA silencing, does not occur with the majority of endogenous genes, although it can happen to a transgene [45; and references therein]. Assuming the lack of transitive RNA silencing, it is possible to induce silencing of a specific copy of a duplicated gene. Targeting a region specific for each copy, e.g., the 3′ untanslated region (UTR), can induce silencing of the gene copy only, whereas targeting a region conserved in duplicated gene copies can induce silencing of the multiple gene copies simultaneously (Figure 3). Such selective RNA silencing was successful in a gene family of rice [125] and this strategy may work for analyzing functional diversification of duplicated genes in any plant species.

An alternative approach to suppress gene expression in plants is the use of artificial miRNAs (Figure 4) (amiRNAs; also called synthetic miRNAs) [38, 126]. This approach involves modification of plant miRNA sequence to target specific transcripts, originally not under miRNA control, and downregulation of gene expression via specific cleavage of the target RNA. Melito *et al*. have used amiRNA to downregulate the leucine-rich repeat transmembrane receptor-kinase gene in soybean [99]. miRNA has been extensively studied in soybean [127-130], information of which may be useful for designing amiRNAs. Because of its specificity, this method will be useful for silencing a limited copy of duplicated genes in soybean.

Induction of TGS by targeting dsRNA to a gene promoter can also be the method of choice. Gene silencing through transcriptional repression can be induced by dsRNA targeted to a gene promoter (Figure 4). However, until recently, no plant has been produced that harbors an endogenous gene that remains silenced in the absence of promoter-targeting dsRNA. We have reported for the first time that TGS can be induced by targeting dsRNA to the endogenous gene promoters in petunia and tomato plants, using a *Cucumber mosaic virus* (CMV)-based vector and that the induced gene silencing is heritable. Efficient silencing depended on the function of the 2b protein encoded in the vector, which facilitates epigenetic modifications through the transport of siRNA to the nucleus [131, 132]. The progeny plants do not have any transgene because the virus is eliminated during meiosis. Therefore, plants that are produced by this system have altered traits but do not carry a transgene, thus constituting a novel class of modified plants [131, 132]. We have also developed *in planta* assay systems to detect inhibition of cytosine methylation using plants that contain a transgene transcriptionally silenced by an epigenetic mechanism [133]. Using these systems, we found that genistein, a major isoflavonoid compound rich in soybean seeds, inhibits cytosine methylation and restores the transcription of epigenetically silenced genes [133]. Whether developing soybean seeds are resistant (or susceptible) to epigenetic modifications is an interesting issue in terms of both developmental control of gene expression and intentionally inducing TGS through epigenetic changes.

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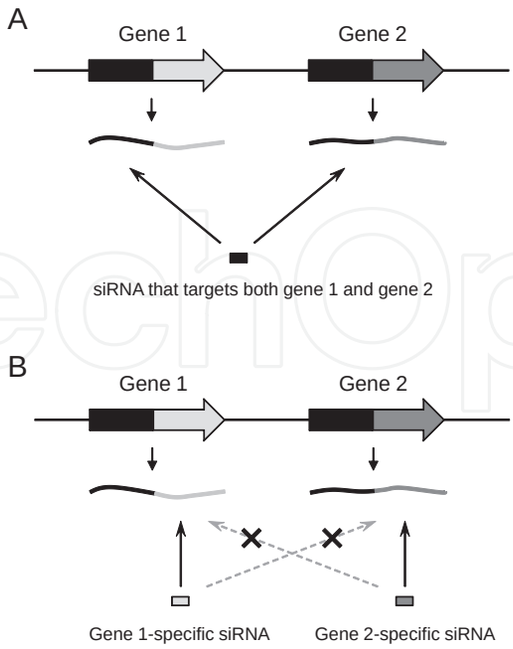


**Figure 3.** Selective RNA silencing of duplicated genes. The gene 1 and gene 2 are produced as a consequence of gene duplication. They share a highly conserved nucleotide sequence in the 5′ region, while they have a different sequence in the 3′ region. When siRNAs corresponding to the conserved region are produced, they can induce RNA degradation of the transcripts from both genes (A). On the other hand, siRNAs corresponding to the 3′ region can induce gene 1-specific or gene 2-specific RNA degradation (B). A combination of these different approaches enables functional analysis of duplicated genes.

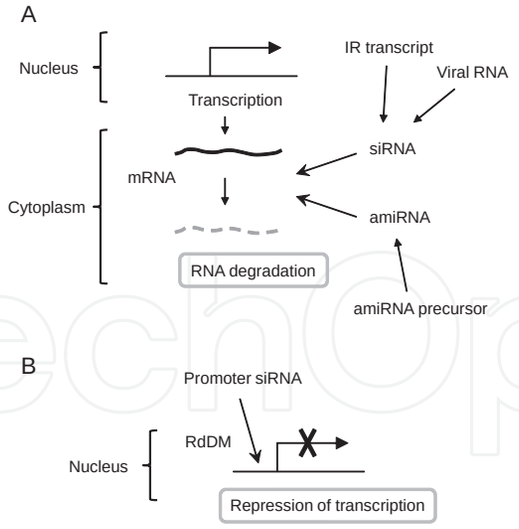


**Figure 4.** Various pathways of RNA silencing that can be intentionally induced to suppress gene expression in plants. Transcripts from transgenes that have an IR sequence of a plant gene segment or viral genomic RNA that carries the segment can form dsRNA. These dsRNAs are subsequently processed into siRNAs in the cytoplasm. Similarly, amiRNA precursors transcribed from the amiRNA gene are processed into amiRNAs. These small RNAs can cause degradation of target gene transcripts, a process termed PTGS (A). When siRNAs corresponding to a gene promoter are produced, they can induce RdDM in the nucleus, thereby TGS of the target gene can be induced (B). Abbreviations: amiRNA, artificial microRNA; PTGS, posttranscriptional gene silencing; RdDM, RNA-directed DNA methylation; TGS, transcriptional gene silencing.

## 8. Differentiation of duplicated genes and induction of RNA silencing

How much sequence difference will be necessary to induce selective RNA silencing? A factor that affects induction of RNA silencing is the extent of sequence identity between the dsRNA that triggers RNA silencing and its target gene. IR-PTGS could be induced by IR-transcripts that can form 98-nt or longer dsRNAs [39]. In VIGS, the lower size limit of the inserted fragments required for inducing PTGS is 23-nt, a size almost corresponding to that of siRNAs [134], and that for inducing TGS is 81-91 nt [135]. Silencing a gene probably requires sequence identity longer than the size of siRNAs between dsRNA and its target, although the efficiency of silencing may depend on the system of silencing induction.

We previously induced *CHS* VIGS in soybean [56]. In soybean seed coats, the *CHS7*/*CHS8* genes, which share 98% nucleotide sequence identity in the coding region, are predominantly expressed among the eight members of the *CHS* gene family [136, 137]. We have induced the silencing using a virus vector that carried a 244-nt fragment of the *CHS7* gene [56]. The *CHS* mRNA levels in the seed coats and leaf tissues of plants infected with the virus were reduced to 12.4% and 47.0% of the control plants, respectively. One plausible explanation for the differential effects of VIGS on these tissues may be that the limited sequence homology (79%-80%) between the *CHS7* and the *CHS1-CHS3* genes, the transcripts of which make up approximately 40% of the total *CHS* transcript content of leaf tissues [137], results in the degradation of the *CHS1-CHS3* transcripts at a lower efficiency than the degradation of *CHS7*/*CHS8* transcripts. Consistent with these results, naturally occurring *CHS* RNA silencing, in which *CHS7*/*CHS8* genes are silenced in seed coat tissues, is thought to be induced by inverted repeat transcripts of a *CHS3* gene segment [110]. In terms of the practical use of transgene-induced RNA silencing, these results suggest that a portion of genes whose sequence identity between duplicated genes is lower than 79%-80% should be chosen as a target for inducing selective RNA silencing.

The naturally occurring RNA silencing of the *CHS* genes in soybean may indicate relationships between diversification of duplicated genes and RNA silencing. Gene duplication can be a cause of RNA silencing because it may sometimes result in the production of dsRNA, which triggers RNA silencing through read-through transcription [114, 115]. In the *CHS* silencing in soybean, the extent of mRNA decrease differs between different copies of the gene family. These observations may indicate that plants use subfunctionalization of duplicated genes as a means to avoid the occurrence of simultaneous silencing of duplicated genes, which may have a deleterious effect on the organism.

## 9. Conclusion and perspectives

RNA silencing has been used as a powerful tool to engineer novel traits or analyze gene function in soybean. Soybean plants that have engineered a metabolic pathway or acquired resistance to diseases have been produced by transgene-induced gene silencing. VIGS has been used as a tool to analyze gene function in soybean. In addition to RNA silencing, site-

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directed mutagenesis using zinc-finger nucleases has been applied to mutagenizing duplicated genes in soybean [138]. Such reverse genetic approaches may be supplemented by forward genetic approaches such as high linear energy transfer radiation-based mutagenesis, e.g., irradiation of ion beam [139] and fast neutron [140]. Similarly, gene tagging systems using maize Ds transposon [141] and rice *mPing* transposon [142] have also been developed in soybean. Aside from using RNA silencing as a tool to engineer novel traits, analysis of mutants in combination with reverse genetic approaches may facilitate the identification of causative gene(s) of the mutation. An interesting feature of RNA silencing is its inducible nature, which allows downregulation of a gene in a tissue-specific manner. This strategy is particularly advantageous for analyzing the function of genes whose mutation or ubiquitous downregulation is lethal. Another feature of RNA silencing is that it allows analysis of biological phenomena that involve the effect of a difference in the mRNA level of the gene. The dependence of pigmentation in soybean pubescence on the mRNA level of the *F3′H* gene has actually been shown by utilizing VIGS [143]. In this regard, selective RNA silencing of duplicated genes may reveal the presence of additive effects of the expression levels of duplicated genes in soybean.

*Abbreviations*

AGO, Argonaute; ALSV, *Apple latent spherical virus*; amiRNA, artificial miRNA; BPMV, *Bean pod mottle virus*; CaMV, *Cauliflower mosaic virus*; CHS, chalcone synthase; CMV, *Cucumber mosaic virus*; dsRNA, double-stranded RNA; EST, expressed sequence tag; F3′H, flavonoid 3′-hydroxylase; IR, inverted repeat; miRNA, micro RNA; MYA, million years ago; PTGS, post-transcriptional gene silencing; RdRP, RNA-dependent RNA polymerase; RISC, RNA-induced silencing complex; RNAi, RNA interference; siRNA, short interfering RNA; SMV, *Soybean mosaic virus*; TFL1, TERMINAL FLOWER1; TGS, transcriptional gene silencing; UTR, untanslated region; VIGS, virus-induced gene silencing

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