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Review

RNA silencing as a tool to uncover gene function and engineer novel traits in soybean

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RNA silencing refers collectively to diverse RNA-mediated pathways of nucleotide-sequence-specific inhibition of gene expression. It has been used to analyze gene function and engineer novel traits in various organisms. Here, we review the application of RNA silencing in soybean. To produce soybean lines, in which a particular gene is stably silenced, researchers have frequently used a transgene that transcribes inverted repeats of a target gene segment. Suppression of gene expression in developing soybean embryos has been one of the main focuses of metabolic engineering using transgene-induced silencing. Plants that have enhanced resistance against diseases caused by viruses or cyst nematode have also been produced. Meanwhile, *Agrobacterium rhizogenes*-mediated transformation has been used to induce RNA silencing in roots, which enabled analysis of the roles of gene products in nodulation or disease resistance. RNA silencing has also been induced using viral vectors, which is particularly useful for gene function analysis. So far, three viral vectors for virus-induced gene silencing have been developed for soybean. One of the features of the soybean genome is the presence of a large number of duplicated genes. Potential use of RNA silencing technology in combination with forward genetic approaches for analyzing duplicated genes is discussed.

Key Words: epigenetic changes, metabolic engineering, post-transcriptional gene silencing, RNA interference, soybean (*Glycine max*), transgene, virus-induced gene silencing.

Introduction

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 post-transcriptional or transcriptional level (reviewed by Brodersen and Voinnet 2006, Vaucheret 2006). 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 (Napoli *et al.* 1990, van der Krol *et al.* 1990). 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 (reviewed by Baulcombe 1996, Wilson 1993). 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 (Metzlaff *et al.* 1997, Waterhouse *et al*. 1998). The potency of dsRNA to induce gene silencing was demonstrated in *Caenorhabditis elegans* by injecting dsRNA into cells in 1998 (Fire *et al.* 1998), and the phenomenon was termed RNA interference (RNAi).

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 (reviewed by Matzke *et al.* 2001). 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 (reviewed by Baulcombe 2004) (Fig. 1). 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) (reviewed by Matzke *et al.* 2009). It has become evident that siRNA plays a role in

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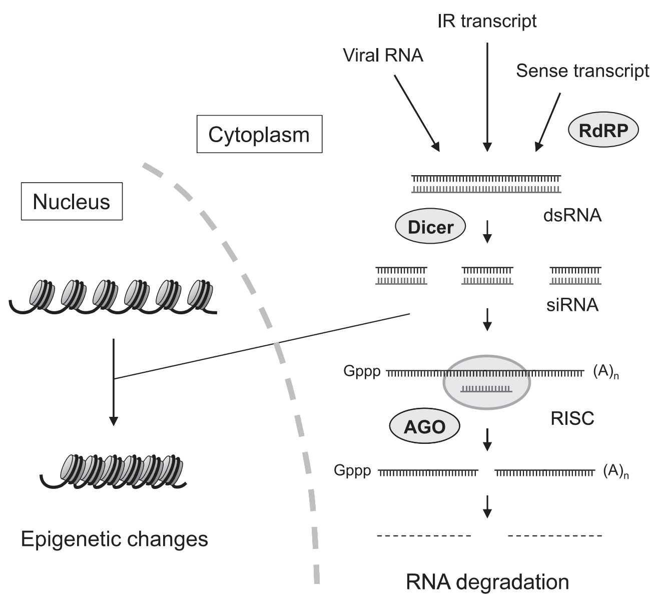

Fig. 1. Pathways of RNA silencing used to engineer novel traits in plants. Posttranscriptional gene silencing is triggered by dsRNA. Transcripts from transgenes that have an IR sequence can form dsRNA. Sense transcripts can produce dsRNA through the synthesis of complementary strand by RdRP. The replication 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. siRNA can also induce epigenetic changes involving DNA methylation and/or changes in histone modification in the nucleus. These changes can convert nucleosomes to a more tightly packed structure, thereby transcription is repressed. Abbreviations: IR, inverted repeat; RdRP, RNA-dependent RNA polymerase; dsRNA, double-stranded RNA; siRNA, short interfering RNA; RISC, RNA-induced silencing complex; AGO, Argonaute.

systemic silencing as a mobile signal (Dunoyer *et al.* 2010, Molnar *et al.* 2010). In addition to siRNA, small RNA molecules called micro RNAs (miRNAs) are also involved in negative regulation of gene expression (reviewed by Mallory and Vaucheret 2006). These gene silencing phenomena that are induced by sequence-specific RNA interaction are collectively called RNA silencing (reviewed by Matzke *et al.* 2004, Voinnet 2002).

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 (reviewed by Baulcombe 2004, Matzke *et al.* 2009, Vaucheret 2006). It can also be used as a tool for analyzing specific gene functions and producing new features in organisms including plants (reviewed by Frizzi and Huang 2010, Kanazawa 2008, Mansoor *et al.* 2006). Here, we review the application of RNA silencing in the genetic analysis and molecular breeding of soybean [*Glycine max* (L.) Merrill].

Methods of transgene-induced RNA silencing in soybean

Engineering novel traits through RNA silencing in soybean has been done using transgenes or virus vectors: examples are listed in Tables 1–3. RNA silencing in some transgenic soybean plants was induced by introducing a transgene that transcribes sense RNA homologous to a gene present in the plant genome, a phenomenon termed co-suppression (Napoli *et al.* 1990). This type of silencing was first discovered in transgenic petunia plants that had silencing of *CHS-A* for chalcone synthase (Napoli *et al.* 1990, van der Krol *et al.* 1990), 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 (Brodersen and Voinnet 2006). 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) (Helliwell and Waterhouse 2005, Wesley *et al.* 2001). 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 (Table 1). 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.

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 (Wagner *et al.* 2011). 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 (Ruiz *et al.* 1998) or by dsRNA injection to *C. elegans* cells (Fire *et al.* 1998), although irregular nuclear processing of primary transcripts associated with PTGS/RNAi has been reported previously (Metzlaff *et al.* 2000). The *FAD2-A1* intron-induced RNA silencing led to the understanding that RNA degradation can take place in the nucleus (Hoffer *et al.* 2011). Although whether RNA degradation in the nucleus is inducible for other genes or in other plants is not known, this phenomenon is intriguing because the involvement of nuclear events has been assumed for amplification of RNA silencing via transitivity (Vermeersch *et al.* 2010) or intron-mediated suppression of RNA silencing (Christie *et al.* 2011).

Transcribing a transgene with a strong promoter tends to induce RNA silencing more frequently than that with a weak promoter (Que *et al.* 1997). For obtaining a higher level of transcription in soybean plants, the *Cauliflower mosaic virus*

Table 1. Metabolic engineering through transgene-induced RNA silencing in soybean

Target gene	Method or construct	Promoter	Transformation	Tissues assayed	Effect	Reference
Fatty acid desaturase gene *FAD2-1*	S-PTGS	β-conglycinin	Particle bombardment	Seed	Increase in oleic acid content	Kinney 1996
β-conglycinin α and α′ subunit genes	S-PTGS	β-conglycinin	Particle bombardment	Seed	Changes in seed protein composition	Kinney *et al.* 2001
β-glucuronidase (*GUS*) gene	S-PTGS	CaMV 35S	Particle bombardment	Leaf and flower	Lack of GUS expression	Reddy *et al.* 2003
Gly m Bd 30 K gene	S-PTGS	β-conglycinin α subunit	Particle bombardment	Seed	Reduced Gly m Bd 30 K	Herman *et al.* 2003
Flavanone 3-hydroxylase gene[a]	S-PTGS	Kti3	Particle bombardment	Seed	Increased isoflavone	Yu *et al.* 2003
Isoflavone synthase genes *IFS1* and *IFS2*	IR-PTGS	FMV	*A. rhizogenes*[c]	Hairy root	Reduced isoflavone and enhanced susceptibility of *P. sojae*	Subramanian *et al.* 2005
Thioredoxin gene	IR-PTGS	CaMV 35S	*A. rhizogenes*[c]	Hairy root	Suppression of root nodule development	Lee *et al.* 2005
Isoflavone synthase genes *IFS1* and *IFS2*	IR-PTGS	Ubiquitin	*A. rhizogenes*[c]	Hairy root	Suppression of root nodule development	Subramanian *et al.* 2006
Myo-inositol-1-phosphate synthase gene	IR-PTGS	CaMV 35S	Particle bombardment	Seed	Absence of seed development and reduced phytic acid	Nunes *et al.* 2006
Senescence-associated receptor-like kinase gene	IR-PTGS	CaMV 35S	*A. tumefaciens*[d]	Leaf	Retarded leaf senescence	Li *et al.* 2006
Delta 15 desaturase gene[b]	IR-PTGS	β-conglycinin α′ subunit	Particle bombardment	Seed	Production of arachidonic acid	Chen *et al.* 2006
Multidrug resistance-associated protein (MRP) ATP-binding cassette (ABC) transporter gene	IR-PTGS	Kti3	Particle bombardment	Seed	Reduced phytic acid	Shi *et al.* 2007
Chalcone synthase gene *CHS6* and isoflavone synthase gene *IFS2*	S-PTGS	CsVMV (CvMV)	*A. rhizogenes*[c]	Hairy root	Reduced isoflavone and coumesterol and increased growth of *F. solani*	Lozovaya *et al.* 2007
Chalcone reductase and isoflavone synthase genes	IR-PTGS	CsVMV (CvMV)	*A. rhizogenes*[c]	Hairy root	Suppression of resistance against *P. sojae* and cell death	Graham *et al.* 2007
Seed oil body protein 24-kDa oleosin gene	IR-PTGS	Oleosin 24-kD isoform A	Particle bombardment	Seed	Changes in seed oil body size and slow growth of the plant	Schmidt and Herman 2008
Fatty acid desaturase gene *GmFAD3*	IR-PTGS	Glycinin	*A. tumefaciens*[d]	Seed	Reduced linolenic acids	Flores *et al.* 2008
Fatty acid desaturase gene *GmFAD2*	IR-PTGS	Lectin	*A. tumefaciens*[d]	Seed	Increased oleic acid	Wang and Xu 2008
Lipoxygenase genes *LOX9* and *LOX10*	IR-PTGS	CaMV 35S	*A. rhizogenes*[c]	Hairy root	No effect on root nodule development	Hayashi *et al.* 2008
Glutathione S-transferase gene *GST9*	IR-PTGS	CsVMV (CvMV)	*A. rhizogenes*[c]	Hairy root	Reduced nitrogenase activity and increased oxidatively damaged proteins	Dalton *et al.* 2009
Ecto-apyrase gene *GS52*	IR-PTGS	FMV	*A. rhizogenes*[c]	Hairy root	Suppression of root nodule development	Govindarajulu *et al.* 2009
FW2.2-like gene *GmFWL1*	IR-PTGS	FMV	*A. rhizogenes*[c]	Hairy root	Suppression of root nodule development	Libault *et al.* 2010
Leucine-rich repeat transmembrane receptor kinase gene	amiRNA[e]	Ubiquitin-3	*A. rhizogenes*[c]	Hairy root	Suppression of root production and no effect on resistance to cyst nematode	Melito *et al.* 2010
Fatty acid desaturase gene *FAD2-1*	Intron IR-PTGS[f]	β-conglycinin α′ subunit	*A. tumefaciens*[d]	Seed	Changes in fatty acid composition	Wagner *et al.* 2011
MYB transcription factor gene *GmMYB176*	IR-PTGS	CaMV 35S	*A. rhizogenes*[c]	Hairy root	Reduced isoflavonoids	Yi *et al.* 2010
Amino aldehyde dehydrogenase gene	IR-PTGS	CaMV 35S	*A. tumefaciens*[d]	Callus	Biosynthesis of 2-acytyl-1-pyrroline	Arikit *et al.* 2011
Glycinin A1bB2 subunit and *FAD-2* genes	IR-PTGS	Glycinin	Particle bombardment	Seed	Changes in seed protein composition	Schmidt *et al.* 2011
β-amyrin synthase genes *GmBAS1* and *GmBAS2*	IR-PTGS	β-conglycinin α′ subunit	Particle bombardment	Seed	Reduced saponin	Takagi *et al.* 2011
Phospholipase D gene SPLDα	IR-PTGS	β-conglycinin α′ subunit	Particle bombardment	Seed	Changes in phospholipid and triacylglycerol composition	Lee *et al.* 2011

[a] The silencing-inducing plasmid was co-bombarded with a plasmid having an expression cassette of the gene encoding a chimeric protein of the maize C1 and R transcription factors.
[b] The silencing-inducing cassette was introduced together with the cassettes that express the delta 5 desatuarase, delta 6 desaturase, and GLELO elongase genes located on the same plasmid.
[c] *Agrobacterium rhizogenes*-mediated root transformation.
[d] *Agrobacterium tumefaciens*-mediated transformation.
[e] Gene silencing induced by artificial microRNA.
[f] Gene silencing induced by transcribing inverted repeats of intron.
Abbreviations: PTGS, posttranscriptional gene silencing; S-PTGS, sense-PTGS; IR-PTGS, inverted repeat-PTGS; CaMV, *Cauliflower mosaic virus*; Kti3, Kunitz trypsin inhibitor 3; FMV, *Figwort mosaic virus*; CsVMV (CvMV), *Cassava vein mosaic virus*; *P. sojae*, *Phytophthora sojae*; *F. solani*, *Fusarium solani*.

(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 are reviewed in another article of this issue (Yamada *et al.* 2012).

Metabolic engineering of soybean plants by transgene-induced RNA silencing

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 (Kinney *et al.* 2001, Schmidt *et al.* 2011), and reducing phytic acids (Nunes *et al.* 2006, Shi *et al.* 2007), saponin (Takagi *et al.* 2011) or allergens (Herman *et al.* 2003) (Table 1). Metabolic engineering has also targeted oil production (Chen *et al.* 2006, Flores *et al.* 2008, Kinney 1996, Lee *et al.* 2011, Schmidt and Herman 2008, Wagner *et al.* 2011, Wang and Xu 2008) (Table 1). These modifications were done by inhibiting a step in a metabolic pathway to decrease a product. On the other hand, RNA silencing can also be used to increase the concentration of a specific metabolite. For example, Yu *et al.* (2003) has produced transgenic soybeans that contain more isoflavone. They induced the activation of genes involved in phenylpropanoid pathway by introducing a transcription factor gene and by blocking a competing branch pathway via co-suppression. Similarly, Artkit *et al.* (2011) demonstrated that RNA silencing of the amino aldehyde dehydrogenase gene induced the biosynthesis of a volatile compound, 2-acetyl-1-pyrroline, in soybean calli, an outcome expected from studies in rice.

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* (Dalton *et al.* 2009, Govindarajulu *et al.* 2009, Hayashi *et al.* 2008, Lee *et al.* 2005, Libault *et al.* 2010, Subramanian *et al.* 2006). The hairy root system was also used for analyzing roles of a MYB transcription factor in isoflavonoid biosynthesis (Yi *et al.* 2010).

Transgene-induced RNA silencing has also been induced in leaf tissues for the β-glucuronidase gene (Reddy *et al.* 2003) or the senescence-associated receptor-like kinase gene (Li *et al.* 2006).

Disease resistance of soybean plants by transgene-induced RNA silencing

Another focus of modifying soybean plants through RNA silencing is resistance against diseases, particularly to those caused by viruses. The effects of gene silencing in plants were first used to develop resistance to viral diseases, even though the mechanism was not clear at the time. Resistance to viruses was achieved by transforming plants with genes or segments of genes derived from viruses and was referred to as pathogen-derived resistance (reviewed by Baulcombe 1996, Goldbach *et al.* 2003, Prins and Goldbach 1996, Wilson 1993). The resistance did not need protein translated from the transgene (Mueller *et al.* 1995, Sijen *et al.* 1996, Smith *et al.* 1994), 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. Use of transgene-induced RNA silencing for plants to acquire resistance against viruses has been reported for various combinations of plants and viruses (reviewed by Baulcombe 1996, Goldbach *et al.* 2003, Mansoor *et al.* 2006). Using this strategy, soybean plants resistant to *Soybean mosaic virus* (SMV; Furutani *et al.* 2006, 2007, Wang *et al.* 2001), or *Soybean dwarf virus* (Tougou *et al.* 2006, 2007) have been produced (Table 2).

In addition to resistance against a virus, transgenic soybean plants resistant to cyst nematode (*Heterodera glycines*) have also been produced using RNA silencing (Steeves *et al.* 2006), 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* (Li *et al.* 2010) or root-knot nematode (*Meloidogyne incognita*) (Ibrahim *et al.* 2011) 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* (Graham *et al.* 2007, Subramanian *et al.* 2005), *Fusarium solani* (Lozovaya *et al.* 2007) or cyst nematode (Melito *et al.* 2010).

VIGS as a powerful tool to analyze gene function

Although transgenes that express a virus-derived gene or gene segment can confer enhanced resistance against virus, plants intrinsically have the ability to cope with viruses. When plants are infected with an RNA virus, dsRNA of the viral genome is degraded by the infected plants (Al-Kaff

Table 2. Enhancement of disease resistance through transgene-induced RNA silencing targeted to pathogens in soybean

Target gene	Construct	Promoter	Transformation method	Reference
Soybean mosaic virus, CP gene and 3′ UTR	cDNA	CaMV 35S	*A. tumefaciens*[a]	Wang *et al.* 2001
Bean pod mottle virus, CP gene	cDNA	CaMV 35S	Particle bombardment	Reddy *et al.* 2001[c]
Soybean dwarf virus, CP gene	cDNA IR	CaMV 35S	Particle bombardment	Tougou *et al.* 2006
Soybean dwarf virus, CP gene	cDNA	CaMV 35S	Particle bombardment	Tougou *et al.* 2007
Soybean mosaic virus, CP gene	cDNA	CaMV 35S	Particle bombardment	Furutani *et al.* 2006, 2007
Cyst nematode (*Heterodera glycines*), major sperm protein gene	cDNA IR	*Arabidopsis* ACT2	Particle bombardment	Steeves *et al.* 2006
H. glycines genes *Cpn-1*, *Y25* and *Prp-17*	cDNA IR	CaMV 35S	*A. rhizogenes*[b]	Li *et al.* 2010
Root-knot nematode (*Meloidogyne incognita*) TP and MSP genes	cDNA IR	FMV	*A. rhizogenes*[b]	Ibrahim *et al.* 2011

[a] *Agrobacterium tumefaciens*-mediated transformation.
[b] *Agrobacterium rhizogenes*-mediated root transformation.
[c] The mechanism of virus resistance in this report could be mainly brought about by the expressed CP protein rather than through RNA silencing. However, we could not exclude the possibility of the involvement of RNA silencing in the phenomenon because no data of the level of viral RNA or *CP* mRNA in the virus-infected plants is presented.
Abbreviations: CP, coat protein; IR, inverted repeat; CaMV, *Cauliflower mosaic virus*; ACT2, actin 2; FMV, *Figwort mosaic virus*; TP, tyrosine phosphatase; MSP, mitochondrial stress-70 protein precursor.

et al. 1998, Covey *et al.* 1997). The dsRNA in the virus-infected cells is thought to be the replication intermediate of the viral RNA (Lu *et al.* 2003) or a duplex structure formed within single-stranded viral RNA (Molnar *et al.* 2005). The viral genomic RNA can be processed into siRNAs, then targeted by the siRNA/RNase complex. In this scenario, if a nonviral segment is inserted in the viral genome, siRNAs would also be produced from the segment. Therefore, if the insert corresponds to a sequence of the gene encoded in the host plant, infection by the virus results in the production of siRNAs corresponding to the plant gene and subsequently induces loss of function of the gene product. This fact led to the use of 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; Kumagai *et al.* 1995, Purkayastha and Dasgupta 2009, Ruiz *et al.* 1998). So far, at least 11 RNA viruses and five DNA viruses were developed as a plant virus vector for gene silencing, as listed previously (Kanazawa 2008). Three vectors are now available in soybean: those based on *Bean pod mottle virus* (BPMV; Zhang and Ghabrial 2006), *Cucumber mosaic virus* (CMV; Nagamatsu *et al.* 2007) and *Apple latent spherical virus* (ALSV; Yamagishi and Yoshikawa 2009) (Table 3).

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 (reviewed by Burch-Smith *et al.* 2004, Lu *et al.* 2003, Metzlaff 2002) and has been used for gene identification via downregulating a candidate gene(s) responsible for a specific phenomenon in soybean. When Nagamatsu *et al.* (2007) tested VIGS on the putative flavonoid 3′-hydroxylase (*F3′H*) gene, the content of quercetin was decreased relative to kaempferol in the upper leaves after viral infection, which indicated that the putative gene actually encodes the F3′H protein. Nagamatsu *et al.* (2007) also demonstrated that VIGS of *CHS* genes resulted in lack of pigmentation in the seed coat tissues. Similarly, VIGS was used to confirm that the *GmTFL1b* gene, a candidate gene for the genetically identified locus *Dt1*, actually controls the determinate habit of soybean plants (Liu *et al.* 2010). 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* (Fu *et al.* 2009, Kachroo *et al.* 2008, Meyer *et al.* 2009, Pandey *et al.* 2011, Singh *et al.* 2011).

Specific features of VIGS

The extent of the induction of silencing is not equivalent between different portions of virus-infected plants because induction of the silencing is associated with propagation of the virus whose extent is often different in different parts of the host plants. This conditional nature of VIGS may have both positive and negative aspects in terms of using the technology for functional genomics. Although the instability may be a negative aspect of VIGS, it may in turn be an advantage by allowing observation of phenotypic changes caused by the dysfunction of a gene whose complete loss of expression is lethal to the plant (Lu *et al.* 2003). In fact, phenotypic changes have been induced by VIGS of the gene for proliferating cell nuclear antigen (Peele *et al.* 2001) and RNA polymerase II (Gosselé *et al.* 2002), for which null mutants cannot be retrieved by conventional or insertional mutagenesis approaches. Similarly, the effect of downregulation of the actin and ribosomal protein genes was detected using VIGS in soybean (Zhang *et al.* 2009).

VIGS has also been applied to genes whose products have a function and/or accumulate during seed development. Whether genes are actually downregulated in developing embryo or donwnregulated in other tissues and the level of transported products is decreased in seeds is intriguing.

Table 3. Virus-induced gene silencing in soybean

Virus	Target gene	Tissues assayed	Effect	Reference
Bean pod mottle virus	Phytoene desaturase gene	Leaf	Photo-bleaching	Zhang and Ghabrial 2006, Zhang *et al.* 2010
	Stearoyl-acyl carrier protein-desaturase gene	Leaf, stem, flower, root and seed	Reduced oleic acid, increased stearic acid and SA and resistance to pathogens	Kachroo *et al.* 2008
	RAR1 and *SGT1*	Leaf	Compromised resistance against SMV and *P. syringae*	Fu *et al.* 2009
	Actin gene	Leaf and root	Severe mosaic, leaf deformation, stunting and reduced SMV accumulation	Zhang *et al.* 2009
	Rebosomal protein genes *Rps6* and *Rps13*	Leaf and root	Very severe foliar symptoms and stunted root growth	
	Mpk4A and *Mpk4B*	Leaf and root	Stunting	
	Sgt1A and *Sgt1B*	Leaf and root	Mild symptom similar to the empty vector-infected control	
	Candidate genes for soybean rust resistance	Leaf	Compromised resistance against *P. pachyrhizi*	Meyer *et al.* 2009
	Candidate genes for soybean rust resistance	Leaf	Compromised resistance against *P. pachyrhizi*	Pandey *et al.* 2011
	Fatty acid desaturase gene *FAD3*	Root, stem, leaf, petiole and seed	Increased seed size and susceptibility to *P. syringae* and BPMV accumulation	Singh *et al.* 2011
Cucumber mosaic virus	Chalcone synthase gene	Seed coat and leaf	Loss of pigmentation in seed coat	Nagamatsu *et al.* 2007
	Chalcone synthase gene	Seed (cotyledon)	Reduced isoflavone	
	Flavonoid 3′-hydroxylase gene	Leaf	Reduced quercetin	
	Flavonoid 3′-hydroxylase gene	Leaf and pubescence	Changes in pubescence color	Nagamatsu *et al.* 2009
	GmTFL1b	Node, pod, and root	Reduced node number	Liu *et al.* 2010
Apple latent spherical virus	Phytoen desaturase gene	Leaf, pod, seed coat and embryo	Photo-bleaching	Yamagishi and Yoshikawa 2009
	Isoflavone synthase 2 gene	Seed (cotyledon)	Reduced isoflavone content	

Abbreviations: SA, salicylic acid; RAR1, required for *Mla12*-mediated resistance; SGT1, suppressor of G2 allele of *skp1*; SMV, *Soybean mosaic virus*; *P. syringae*, *Pseudomonas syringae*; Mpk, mitogen-activated protein (MAP) kinase; TFL, terminal flower; *P. pachyrhizi*, *Phakopsora pachyrhizi*; BPMV, *Bean pod mottle virus*.

Yamagishi and Yoshikawa (2009) showed that RNA silencing of the phytoene desaturase and isoflavone synthase 2 genes actually occurs in soybean embryos, resulting in photobleaching and a decrease in isoflavone content, respectively, by the ALSV vector. A decrease in isoflavone content in soybean embryos has also been induced by the CMV vector (Nagamatsu *et al.* 2007).

Modification and optimization of VIGS in soybean

When VIGS is used to analyze the function of a gene, viral infection itself might be a problem depending on the target gene. Symptoms of virus infection indicate that gene expression in the infected cells has been affected. If a gene with expression affected by viral infection is chosen as the target of VIGS, the effect of VIGS might not appear as a specific effect caused by the sequence-specific degradation of the RNA, but a nonspecific effect of the viral infection might also be involved. Accordingly, efforts are sometimes needed to reduce the extent of nonspecific effects of viral infection and simultaneously efficiently induce VIGS. In this respect, when a new combination of plant species and virus vector is used, it is often necessary to control the efficiency of viral infection and symptom production to optimize the induction of VIGS (Kanazawa 2008).

Symptomless viral infection of soybean has been achieved using CMV (Nagamatsu *et al.* 2007) and ALSV (Yamagishi and Yoshikawa 2009). In case of CMV infection, a pseudorecombinant virus that consists of RNA components derived from different CMV strains were used to establish symptomless infection of the virus (Nagamatsu *et al.* 2007). Moreover, VIGS that accompanies neither severe viral symptoms nor outward phenotypic changes has been achieved by targeting the *F3′H* gene in soybean, while the flavonoid content was successfully modified by the VIGS (Nagamatsu *et al.* 2007).

For infection of soybean plants with RNA viruses, tissues of young plants are inoculated with *in-vitro*-generated transcripts of the viral genome or with the sap or RNA extracted from an infected leaf of other plants. Infection of soybean plants with ALSV was done by the following method. Plasmids containing ALSV cDNA were inoculated onto *Chenopodium quinoa* leaves. RNA extracted from the leaves was then introduced into cotyledons of soybean seedlings through particle bombardment-mediated delivery (Yamagishi and Yoshikawa 2009). For infection of soybean plants with CMV, the virus was first propagated in *Nicotiana benthamiana* plants: leaves of *N. benthamiana* were rub-inoculated with the *in-vitro*-generated transcripts of viral cDNA. Then, the primary leaves of soybean plants were inoculated with the sap from an infected leaf of the *N. benthamiana* plant (Nagamatsu *et al.* 2007). Infection of soybean plants with *in-vitro*-generated transcripts was also possible (Kanazawa *et al.*, unpublished data). A similar method was also used for infection of BPMV (Zhang and Ghabrial 2006). The method of infection of soybean plants with BPMV has been modified as follows. Zhang *et al.* (2009) made DNA constructs in which the cDNA of the BPMV RNAs is transcribed under the control of the CaMV 35S promoter. They achieved infection of soybean plants with the virus through particle bombardment-mediated delivery of these DNA constructs to facilitate VIGS experiments. Zhang *et al.* (2010) also modified the BPMV vector and overcame its constraint that target sequences must be expressed as fusion proteins with a viral polypeptide.

Use of viral infection as a tool to “diagnose” an RNA-silencing-induced phenotype

Another interesting aspect of the use of viruses for the study of RNA silencing in plants is the function of a virus-encoded suppressor protein of RNA silencing. 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 (reviewed by Silhavy and Burgyan 2004). It has been known that the lack of brown pigmentation in the seed coat of soybean is caused by naturally occurring *CHS* RNA silencing (Senda *et al.* 2004, reviewed by Senda *et al.* 2012). When a soybean plant that has a yellow seed coat is infected with CMV, the seed coat restores pigmentation (Senda *et al.* 2004). 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 (Della Vedova *et al.* 2005) and petunia (Koseki *et al.* 2005), both of which have phenotypic changes through naturally occurring RNA silencing of an endogenous gene.

RNA silencing as a tool to understand regulatory mechanisms of biological phenomenon associated with mRNA level of a gene

RNA silencing of a particular gene is also useful for analyzing biological phenomena, in particular those involving the effect of a difference in the mRNA level of the gene. For example, the regulatory mechanisms of pigmentation in soybean pubescence was analyzed using VIGS of the *F3′H* gene, whose function is necessary for pigmentation of soybean pubescence. Silencing did not result in lack of pigmentation when plants were grown in normal greenhouse conditions, but plants lacked pigmentation when grown in controlled conditions; the steady-state mRNA level of the *F3′H* gene was reduced to ca. 5% of that of greenhouse-grown plants (Nagamatsu *et al.* 2009). VIGS in the controlled conditions resulted in a further decrease in the mRNA level, which led to the discovery that a threshold mRNA level of the *F3′H* gene was associated with the pigmented pubescence (Nagamatsu *et al.* 2009).

Future prospects of the use of RNA silencing in soybean

1. Stability and heritability of RNA silencing

Induction of transgene-mediated RNA silencing can be affected by various factors such as structure, copy number, or expression level of the transgene, environmental conditions or developmental stages of the plant (Majewski *et al.* 2009, and references therein). In addition, induction of transgene-mediated RNA silencing can be destabilized during cell proliferation and appears to be re-initiated in each generation (Furutani *et al.* 2007, Mitsuhara *et al.* 2002). However, transgene-mediated RNA silencing can induce a strong, tissue-specific or ubiquitous silencing and is suitable for producing plants in which one or more genes are stably silenced in the presence of the transgene as far as the transgene is capable of inducing the silencing. On the other hand, because of elimination of viruses during meiosis, VIGS is basically transient and is confined to the plants in which the virus is inoculated. An exceptional VIGS vector applicable to soybean is ALSV: it can transmit to the next generation and induce silencing across generations (Yamagishi and Yoshikawa 2009).

Heritable silencing can be induced in plants via induction of epigenetic changes by CMV, although the virus does not transmit to the subsequent generation (Kanazawa *et al.* 2011a, 2011b). Gene silencing through transcriptional repression can be induced by dsRNA targeted to a gene promoter (reviewed by Matzke *et al.* 2004). 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 CMV-based vector and that the induced gene silencing is heritable (Kanazawa *et al.* 2011a). 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 (Kanazawa *et al.* 2011a). The efficiency of promoter-targeted silencing also depends on features of promoter RNA segments (e.g., length and nucleotide composition) (Otagaki *et al.* 2011). An advantage of the RNA-mediated TGS using the CMV vector is that the progeny plants do not have any transgene because the virus is eliminated during meiosis. Plants that are produced by this system have altered traits but do not carry a transgene, thus constituting a novel class of modified plants (Kanazawa *et al.* 2011b). Because of the availability of the vector in soybean, VIGS can potentially be used for producing such novel class of plants in soybean as well.

2. RNA silencing as a tool to analyze duplicated genes

One feature of the soybean genome is the presence of a large number of duplicated genes. 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 (Shoemaker *et al.* 1996), with nearly 75% of the genes present in multiple copies (Schmutz *et al.* 2010). In addition, genes in the soybean genome are sometimes duplicated in tandem (e.g., Kong *et al.* 2010, Matsumura *et al.* 2005, Schlueter *et al.* 2008, Yoshino *et al.* 2002). Our recent studies have indeed shown functional redundancy of duplicated genes in soybean (Kanazawa *et al.* 2009, Liu *et al.* 2008). 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.

In addition, 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. In plants, siRNAs promote production of secondary siRNAs from 5′ and/or 3′ 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 (reviewed by Voinnet 2008). 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 (Vermeersch *et al.* 2010, 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′ 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. Such selective RNA silencing was successful in a gene family of rice (Miki *et al.* 2005) and this strategy may work for analyzing functional diversification of duplicated genes in any plant species. Considering the presence of a large number of gene-level duplication and/or chromosomal segmental duplication, it should also be noted that naturally occurring gene silencing may be discovered in soybean in the future in addition to *CHS* silencing, which is manifested as a visibly altered phenotype (Senda *et al.* 2004, 2011).

3. Potential targets and methods

Tolerance to abiotic stress and fertility control through RNA silencing have been reported for various plants (Mansoor *et al.* 2006). These traits can be a future target of RNA silencing in soybean. Attempts to induce RNA silencing only in seeds will increase because genes responsible for the synthesis of seed components are sometimes essential for normal vegetative growth, whose downregulation in nonseed tissues might have deleterious effects on plant growth. A typical example is the embryo-specific silencing of a transporter gene, which results in reduction of phytic acid content in soybean seeds without inducing undesirable agronomic characters associated with phytic acid reduction (Shi *et al.* 2007).

A recently introduced approach to suppress gene expression in plants is the use of artificial miRNAs (amiRNAs; also called synthetic miRNAs; reviewed by Frizzi and Huang 2010, Ossowski *et al.* 2008). This approach involves modification of plant miRNA sequence to target specific transcripts, originally not under miRNA control, and downregulation of gene expression via specific cleavage of the target RNA. This method has been applied to target viral RNA (Niu *et al.* 2006) and transcripts of endogenous genes in plants (Alvarez *et al.* 2006, Schwab *et al.* 2006). In soybean, Melito *et al.* (2010) have used amiRNA to downregulate the leucine-rich repeat transmembrane receptor-kinase gene. Considering that miRNA has been extensively studied in soybean (e.g., Song *et al.* 2011), amiRNA can be the method of choice for RNA silencing in soybean. Because of its specificity, this method will be useful for silencing a limited copy of duplicated genes.

Such a reverse genetic approach may also be supplemented by forward genetic approaches already done in soybean such as high linear energy transfer radiation-based mutagenesis, e.g., irradiation of ion beam (Arase *et al.* 2011) and fast neutron (Bolon *et al.* 2011), which potentially bring about a large deletion in the genome. Similarly, a gene tagging system using the Ds transposon (Mathieu *et al.* 2009) has also been developed in soybean. A combination with these different approaches will increase the applicability of RNA silencing.

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