New Hairpin RNAi Vector with *Brassica rapa* ssp. *pekinensis* Intron for Gene Silencing in Plants

Gi-Ho Lee†, Gang-Seob Lee‡, and Young-Doo Park†*

1Department of Horticultural Biotechnology, Kyung Hee University, Yongin 17104, Korea
2Biosafety Division, National Academy of Agricultural Science, Rural Development Administration, Jeonju 54874, Korea

*Corresponding author: ydpark@khu.ac.kr

These authors contributed equally to this work.

Abstract

Homology-specific transcriptional and post-transcriptional silencing, an intrinsic mechanism of gene regulation in most eukaryotes, can be induced by anti-sense, co-suppression, or hairpin-based double-stranded RNA. Hairpin-based RNA interference (RNAi) has been applied to analyze gene function and genetically modify crops. However, RNAi vector construction usually requires high-cost cloning steps and large amounts of time, or involves methods that are protected by intellectual property rights. We describe a more effective method for generating intron-spliced RNAi constructs. To produce intron-spliced hairpin RNA, an RNAi cassette was ligated with the first intron and splicing sequences of the *Brassica rapa* ssp. *pekinensis* histone deacetylase 1 gene. This method requires a single ligation of the PCR-amplified target gene to SpeI-NcoI and SacI-BglII enzyme sites to create a gene-specific silencing construct. We named the resulting binary vector system *pKHi* and verified its functionality by constructing a vector to silence *DIHYDROFLAVONOL 4-REDUCTASE* (DFR), transforming it into tobacco plants, and confirming *DFR* gene-silencing via PCR, RT-qPCR, and analysis of the accumulation of small interfering RNAs. Reduction of anthocyanin biosynthesis was also confirmed by analyzing flower color of the transgenic tobacco plants. This study demonstrates that small interfering RNAs generated through the *pKHi* vector system can efficiently silence target genes and could be used in developing genetically modified crops.

Additional key words: chinese cabbage, *DFR*, gene silencing, ishpRNA, small RNA

Introduction

Hairpin-based RNA interference (hpRNAi) is a sequence-specific gene silencing strategy used for transcriptional or post-transcriptional gene silencing and functions by changing chromatin modifications at the target locus or by degrading or blocking the translation of homologous mRNAs in the cytoplasm, respectively (Wassenegger, 2000; Aufsatz et al., 2002). For example, RNAi can regulate gene expression in plants via double stranded RNA-mediated DNA sequence methylation. Many studies have used hairpin-based RNAi in the functional analysis of genes and for the development of genetically modified...
crops (Kim et al., 2012; Mo et al., 2016; Park et al., 2016; Park et al., 2017). Over the past 20 years, functional genomics has played a key role elucidating the complex mechanisms of gene expression networks and the regulation of physiological and biochemical processes.

Large-scale genome studies have used next-generation sequencing to analyze the mechanisms of metabolic processes, and identify the genes underlying ecotype variation. These large-scale approaches generated genome sequences of Arabidopsis (Arabidopsis Genome Initiative, 2000; Gan et al., 2011), Oryza sativa (rice; 3,000 Rice Genomes Project, 2014), and Brassica rapa ssp. pekinensis (Chinese cabbage; Wang et al., 2011). These studies have also demonstrated the complex relationships plant genes have with changes in signal transduction and shown that a large proportion of genes have unknown functions. Subsequently, reverse genetic techniques like RNAi have been widely used to study gene function, develop genetically modified crops, and modulate plant metabolic pathways. Examples include the biosynthesis of stearic and oleic acids in cotton (Liu et al., 2002; Abdurakhmonov et al., 2016), the biological regulation of soybean oil quality (Clemente and Cahoon, 2009; Haun et al., 2014), and the synthesis of artemisinin in Artemisia annua (Kumar et al., 2016).

Hairpin RNA (hpRNA) technology, in which foreign DNA molecules are designed to express single-or double-strand self-complementary RNA, can also be used to trigger transcriptional and post-transcriptional gene silencing (Wang and Waterhouse, 2000). The hpRNA binary vectors pHANNIBAL and pKANNIBAL, which use traditional recombinant DNA methods to clone hpRNA, have been commonly used as vectors to transform plants with gene-specific hpRNA constructs. By contrast, the pHELLSGATE and pSTARGATE vector systems (CSIRO Plant Industry, Canberra, Australia) use the Gateway recombination technology (Wesley et al., 2001; Helliwell et al., 2002; Helliwell and Waterhouse, 2003). The p# 7GWIWG2(I) and p# 7GWIWG2(U) binary vector systems, which also use Gateway recombination, have been widely used and can be easily purchased from the Flanders Interuniversity Institute for Biotechnology (https://gateway.psb.ugent.be). However, these vector systems are only available for academic research, and the development of new RNAi vector systems are necessary for commercializing hpRNA-based genetically modified crops.

The present study developed the pKHi plant transformation vector system for transcriptional and post-transcriptional gene silencing studies and potential commercialization. This study also tested the vector system by constructing a DFR gene-silencing vector for the transformation of tobacco plants (Nicotiana tabacum). This vector system could provide a more effective method for generating intron-spliced hairpin RNA (ishpRNA) constructs for basic and applied research. Moreover, our new RNAi vector system could be used to facilitate the commercialization of genetically modified crop.

Materials and Methods

Plant Genomic DNA Isolation

Briefly, leaf tissues (0.5 g) were harvested from six-week old Chinese cabbage (Brassica rapa ssp. pekinensis) plants, and genomic DNA was isolated using 2×CTAB extraction buffer (2.0% CTAB, 0.1 M Tris-Cl / pH 8.0, 1.4 M NaCl, 20 mM EDTA, 1% β-mercaptoethanol, 2% polyvinylpyrrolidone, and 1% sodium bisulfite) with a modified version of the previously described CTAB protocol (Doyle and Doyle, 1987).
**BrHD1 Intron Isolation and RNAi Cassette Construction**

The first intron region of the *B. rapa histone deacetylase 1* gene (*BrHD1*) was amplified using PCR (Maxime i-Star Taq, PCR PreMix; #25165, iNTRON Biotechnology Inc, Seongnam, Korea) with the primers HD-Intron-F (5′-CTCTTAAGCAGCATG-AGTTTGCTC-3′) and HD-Intron-R (5′-AGATCTACGAGATACGACTG-3′). In order to build a newISHpRNAi vector system, a hairpin RNAi cassette was constructed by cloning the first *BrHD1* intron region and attaching it to widely used enzyme sites. The enzyme sites, NcoI-Sacl and BglII-Spel-BstEII, were linked to the 5′ and 3′ ends of the first intron region, respectively. The front enzyme site consisted of Neol-Sacl plus (5′-CCATGGCAGCGGAGAATTCGATTGAGCTC-3′) and Neol-Sacl minus (5′-GAGCTCAATCGAATTCGCGCAGATG-3′), and the end enzyme site consisted of BglII-Spel-BstEII plus (5′-AGATCTATACGAGATGCTGCAGGACC-3′) and BglII-Spel-BstEII minus (5′-GTCGACGAGATGCTGCAGGACC-3′). The entire enzyme site was ligated as described by Siu et al. (2008). 

**Construction of RNAi Binary Vector System**

In order to build a newISHpRNAi vector system, a hairpin RNAi vector was constructed by modifying the *pCAMBIA* 3301 vector (CAMBIA, Canberra, Australia). At first, *pCAMBIA* 3301 was digested with EcoRI and NcoI to delete the Sacl enzyme site from the multi-cloning site (MCS) region. Then, a 606-bp genomic DNA fragment that contained the cauliflower mosaic virus (*CaMV*) 35S promoter was amplified using PCR and the primers 35S-EcoRI-F (5′-GAATTCTCATGGAGTCAAAAGA-TTC-3′) and 35S-R (5′-CTCTTAACGAGAAATTGAGGAGA-3′), which added EcoRI site to the 5′ end and contained NcoI site to the 3′ end of the amplified MCS-*CaMV* 35S promoter sequence (Fig. S1B). The resulting *pCAMBIA* 3301-m vector was digested with NcoI and BstEII to remove the β-glucuronidase (GUS) region, after which the intron fragment with the NcoI and BstEII sites was inserted. The intron is flanked by NcoI and SacI sites on the 5′ side and by BglII, SpeI, and BstEII site on the 3′ side. 

The entire GUS coding sequence was replaced with the RNAi cassette in the modified *pCAMBIA* 3301 ( *pCAMBIA* 3301-m in the present study). As a result, the RNAi cassette was inserted between the *CaMV* 35S promoter and the nopaline synthase terminator. The constructed vector was named pkHi (Fig. S1C) and confirmed by enzyme digestion and sequencing analysis (Macrogen Co., Seoul, Korea). 

**Construction of the BrDFR Gene Silencing Vector using the pkHi Vector System**

To verify the *pkHi* vector system, a vector to silence the *B. rapa* gene encoding dihydroflavonol 4-reductase (*BrDFR*, GenBank ID: AY567978) was constructed. To induce *DFR* gene silencing, a 473-bp fragment of *BrDFR* was amplified using PCR with the primers DFRiTF (5′-ACTAGTCCATGGGAAATCTAAAAGATCC-3′) and DFRiTR (5′-AGATCTCAGCTCAAAGACTCGAGATAG-3′), which added SpeI-NcoI and Sacl-BglII sites to the 5′ and 3′ ends of the amplified DNA that will produce the hpRNA. To prevent off-target effects, the primer set was analyzed using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast; Ye et al., 2012). PCR was performed in 20-µL reaction mixtures with hot-start Taq. The amplified *BrDFR* DNA in the pGEM-T easy vector was digested using NcoI-Sacl for sense orientation and BglII-Spel for antisense orientation, and the digested *BrDFR* DNA fragments were inserted into the *pkHi* vector (Fig. S1D). The resulting *DFR* gene silencing vector (*DFRi*) was confirmed using enzyme digestion and sequencing (Macrogen Co., Seoul, Korea) and was then introduced into *Agrobacterium tumefaciens* strain LBA4404 using a modified freeze-thaw method (Jyotishwaran et al., 2007).
**Agrobacterium-mediated Transformation of Tobacco**

The transformation of tobacco (*Nicotiana tabacum* L. ‘Petit Havana SRI’) was performed as described previously (Lee et al., 2008). To identify successfully transformed *DFRi* transgenic lines, genomic DNA was isolated as described above, and a 384-bp *Bar* fragment was amplified using PCR with the primers Bar-F (5′-GTAGAGCGTGAGCCCCAGT-3′) and Bar-R (5′-TACCATGAGCCCAGAAGAC-3′).

**Confirmation of Tobacco Transformation**

To isolate total RNA from the *DFRi* transgenic tobacco plants, 200 mg of leaf tissue was ground in liquid nitrogen and extracted using a plant-specific total RNA extraction kit (MiniBEST Plant RNA Extraction Kit, #9769A; TaKaRa, Otsu, Japan), according to the manufacturer’s instructions. RT-PCR and RT-qPCR were performed as described previously (Lee et al., 2010), and the same primers were used for PCR genotyping and quantitative real-time RT-PCR: 5′-TATGAGCACCCAAAGGCAGAGG-3′ and 5′-CGGCCATTTTCTCTTGACCACCATC-3′ for NtDFR and 5′-TCCACATGCTATTTCCGTTT-3′ and 5′-CCCTGACAATTTCGCCGCTCA-3′ for the control NtActin.

**Analysis of *DFRi* Vector-mediated siRNAs**

Small RNAs were isolated from the transgenic plants using the mirVana miRNA isolation kit (# AM1560; Ambion Inc., TX, USA) according to the manufacturer’s instructions, and small RNA detection was performed as described by Pall and Hamilton (2008). To measure siRNA accumulation of *DFRi* transgenic tobacco, small RNAs were isolated from leaves of transgenic tobacco and separated using denaturing polyacrylamide gel electrophoresis (dPAGE) with 15% gels that contained 7 M urea buffered with 20 mM MOPS-NaOH (pH 7.0), blotted onto a nylon membrane, and hybridized to [γ-32P]dATP-labeled *DFR* probes. The DNA probes corresponded to nucleotides in the *DFR* gene that were PCR amplified using the primers *DFR*-RNAitotal-F (5′-CTAGCATGGAATCTAAGGATCCC-3′) and *DFR*-RNAitotal-R (5′-GATCAGCTAGAACTCGGAGAT-AG-3′).

**Phenotypic Analysis**

The confirmed transgenic tobacco lines were cultivated in the greenhouse to observe their phenotypes. When the plants were in full bloom, the petal color of each plant was measured, using LAB color space. Components of the CIE LAB color scale are lightness (*L*°), ranging from 0 (black) to 100 (white); *a*°, for which negative numbers denote greenness and positive numbers denote redness; and *b*°, for which negative numbers denote blueness and positive numbers denote yellowness (Childers and Brecht, 1996).

**Results and Discussion**

**Construction of a Novel IshpRNAi Vector System**

A single self-complementary hpRNA was constructed, in order to develop a novel siRNA system for gene silencing. In previous studies, the loop structure of the hairpin RNA vector contained an 800-nucleotide spacer fragment with an intron or a
non-splicing intron fragment, which resulted in 96% and 90% target gene silencing, respectively (Smith et al., 2000; Wesley et al., 2001). The demonstrated efficacy of gene-silencing constructs in plants showed that the addition of intron sequences to splicing signals in hairpin structures increases hairpin structure stability and loop formation by endogenous splicing mechanisms (Smith et al., 2000). Moreover, RNAi efficiency is highly dependent on the length of the stem region. The most commonly used stem length ranges from about 200 to 500 bp, and stems longer than 1.1 kb often result in inefficient silencing (Heilersig et al., 2006; Hirai and Kodama, 2008). RNAi constructs containing a sense direction intron and intron splicing can efficiently silence a target gene; therefore, an intron-spliced hairpin RNA was successfully developed in this study.

The pKHi binary vector system reported here was derived from pCAMBIA 3301, the backbone of which derived from the pPZP vector (Hajdukiewicz et al., 1994) containing both bacterial and plant selection markers: neomycin phosphotransferase gene (NPTII) conferring kanamycin resistance and the phosphinothricin N-acetyltransferase gene (Bar) conferring phosphinothricin resistance, respectively. The ishRNA vector system (pKHi), into which gene fragments can be introduced, contains GT-AG splicing signal sequences confirmed by sequencing (Figs. 1A and 1B). Finally, the constructed pKHi vector clone containing the desired insert was selected by restriction enzyme mapping (Figs. 1C and 1D). In this vector system, the construction of gene-silencing ishRNA only requires a single ligation of PCR-amplified target silencing region to Spel-Ncol and SacI-BglII enzyme sites (Fig. 1E).

**Verification of the pKHi/Vector System by Silencing DFR**

To verify the pKHi vector system, we targeted the DFR gene and analyzed the effects of a DFRi transgene in tobacco plants using the pKHi backbone. DFR is an important enzyme in the flavonoid synthetic pathway (Holton and Cornish, 1995). Overexpression of BrDFR in transgenic tobacco significantly affected petal color due to increased cyanidin and pelargonidin biosynthesis in the anthocyanin pathway (Lee et al., 2008). For expression of DFR siRNAs in tobacco plants, the ishRNA construct was designed using an internal 473-bp region of DFR as the hpRNA stem to be transcribed and form dsRNA (Fig. 2A). The target DFR siRNA fragment was introduced on either side of the first BrHD1 intron RNAi cassette, and the ishRNA vector clone with the correct insert was selected by restriction enzyme mapping (Figs. 2B and 2C). Afterward, the tobacco plants were transformed with the constructed DFRi vector and confirmed by PCR analysis with four independent transgenic tobacco plants used for molecular and phenotypic analyses (Figs. 2D and 2E).

We used RT-qPCR to confirm the expression of DFR in DFRi-transformed tobacco plants and measured expression levels by RT-qPCR (Figs. 3A and 3B). Transgenic plants exhibited significantly lower (4.3- to 6.67-fold) DFR mRNA accumulation than wild-type plants. We conclude that the transgenic tobacco plants exhibited significantly decreased DFR expression demonstrating the effectiveness of the pKHi vector system.

To investigate whether the construct could induce accumulation of siRNA molecules in transgenic tobacco, small RNA detection was conducted using northern blot analysis. Until fairly recently, Northern blot analysis for small RNA detection has been primarily used in Arabidopsis (Ye et al., 2016), rice (Song and Cao, 2016), and maize (Xia et al., 2016). The present study also used chemical (EDC) cross-linking of small RNA to a nylon membrane, which enhanced small RNA hybridization (Pall and Hamilton, 2008). Three selected transgenic tobacco lines exhibited accumulation of 21-nt siRNAs (Fig. 3C), whereas one early senescent transgenic line (DFRi-2) did not. These results indicate the 21-nt DFR siRNAs in transgenic tobacco trigger the degradation of NdFR mRNA by post-transcriptional gene silencing, which occurs in the cytoplasm and involves dsRNA degradation (Aufsatz et al., 2002).
For phenotypic analyses, the transgenic plants exhibited a change in petal color (from red to light pink) compared to the non-transgenic tobacco plants (Fig. 4A). The measured values of CIE Lab color parameters $L^*$, $a^*$, and $b^*$ were 1.23, 1.41, and 3.00-fold greater for transgenic tobacco plants than for non-transgenic plants, respectively (Fig. 4B). Petal color was shifted to light green and light blue, which is perceived by the human eye as a change to light pink. In other words, these phenotypes indicated
a significant negative tendency of the $a^*$ and $b^*$ values in DFRi transgenic plants. A plausible interpretation of these results is lower red pigment content compared to non-transgenic tobacco. Previous studies in Gossypium species (cotton; Tan et al., 2013), Rosa hybrida (rose; Schmitzer et al., 2010), Lycoris longituba (lycoris; He et al., 2011), and Myrica rubra (Chinese bayberry; Niu et al., 2010) have reported changes in petal color are correlated with anthocyanin accumulation. These results indicate that DFRi vector-mediated DFR gene-silencing in transgenic tobacco plants accumulating 21-nt siRNAs exhibit lower anthocyanin biosynthesis.

The present study describes a more effective method for generating ishRNA constructs for transcriptional and post-transcriptional gene silencing. Moreover, the pKHi vector system is unconstrained by either domestic or international intellectual property rights. Therefore, the system is free from obstacles to commercialization of RNA-silenced genetically modified crop development.
New Hairpin RNAi Vector with *Brassica rapa* ssp. *pekinensis* Intron for Gene Silencing in Plants

Horticultural Science and Technology

Fig. 3. *DFRi* effectively silences *DFR* in transgenic tobacco. (A) RT-PCR confirmation of *DFR* expression in wild-type and transgenic tobacco plants. SRI, non-transgenic tobacco; *DFRi*-1–4, transgenic tobacco lines. (B) Analysis of *BrDFR* gene expression by RT-qPCR analysis. Error bars indicate standard deviation. (C) Accumulation of the *DFR* siRNA in selected transformants and in non-transgenic tobacco. P, ssRNA marker (21 nt).

Fig. 4. Anthocyanins in flowers are reduced in *DFRi* transgenic tobacco plants. Flower phenotypes of *DFR* gene-silenced and non-transgenic tobacco plants. (A) Phenotype analysis. (B) Distribution of petals color based on trivariante (*L*, *a*, and *b*) CIE-Lab color space.

Literature Cited


