RESEARCH ARTICLE https://doi.org/10.12972/kjhst.20170035

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

Gi-Ho Lee[1†], Gang-Seob Lee[2†], and Young-Doo Park[1*]

[1]Department of Horticultural Biotechnology, Kyung Hee University, Yongin 17104, Korea
[2]Biosafety 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

Received: December 5, 2016
Revised: March 11, 2017
Accepted: March 24, 2017

HORTICULTURAL SCIENCE and TECHNOLOGY
35(3):323-332, 2017
URL: http://www.kjhst.org

pISSN : 1226-8763
eISSN : 2465-8588

This work was carried out with the support of "Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ01112301)" Rural Development Administration, Republic of Korea.

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*(*II*) 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-AGGTTTGTC-3′) and HD-Intron-R (5′-AGATCTAACGTACAGAACACGCTG-3′). In order to build a new ishpRNAi 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-SacI and BglII-SpeI-BstEII, were linked to the 5′ and 3′ ends of the first intron region, respectively. The front enzyme site consisted of NcoI-SacI_plus (5′-CCATGGCGGCCGCGGGAATTCGATTGAGCTC-3′) and NcoI-SacI_minus (5′-GAGCTCAATCGAATTCCCGCGGCCGCCATGG-3′), and the end enzyme site consisted of BglII-SpeI-BstEII_plus (5′-AGATCTATCACTAGTGGTGACC-3′) and BglII-SpeI-BstEII_minus (5′-GGTCACCACTAGTG-ATAGAT CT-3′; Fig. S1A). The oligonucleotides were ligated as described by Siu et al. (2008).

Construction of RNAi Binary Vector System

In order to build a new ishpRNAi 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 SacI 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′-GAATTCTCATGGAGTCAAAGA-TTC-3′) and 35S-R (5′-CCTAACCAAGAAAATGAAGGAGA-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 (Macrogene 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 *DFRi*-TF (5′-ACTAGTCCATGGGAATCTAA GGATCCC-3′) and *DFRi*-TR (5′-AGATCTGAGCTCA GAAACTCGGAGATAG-3′), which added SpeI-NcoI and SacI-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-SacI for sense orientation and BglII-SpeI 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 (Macrogene 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′-GTAGAGCGTGGAGCCCAGT-3′) and Bar-R (5′-TACCATGAGCCCAGAACGAC-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′-TATGAGCACCCAAAG GCAGAGG-3′ and 5′-CGGCCATTTCTCTTGGACCATC-3′ for Nt*DFR* and 5′-TCCCACATGCTATTCTCCGCTT-3′ and 5′-CCCTGACAATTTCCCGCTCA-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-^{32}P] 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′-GATCAGCTAGAAACTCGGAGAT -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 ishpRNA 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 ishpRNA only requires a single ligation of PCR-amplified target silencing region to SpeI-NcoI 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 ishpRNA 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 ishpRNA 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-PCR 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 *NtDFR* mRNA by post-transcriptional gene silencing, which occurs in the cytoplasm and involves dsRNA degradation (Aufsatz et al., 2002).

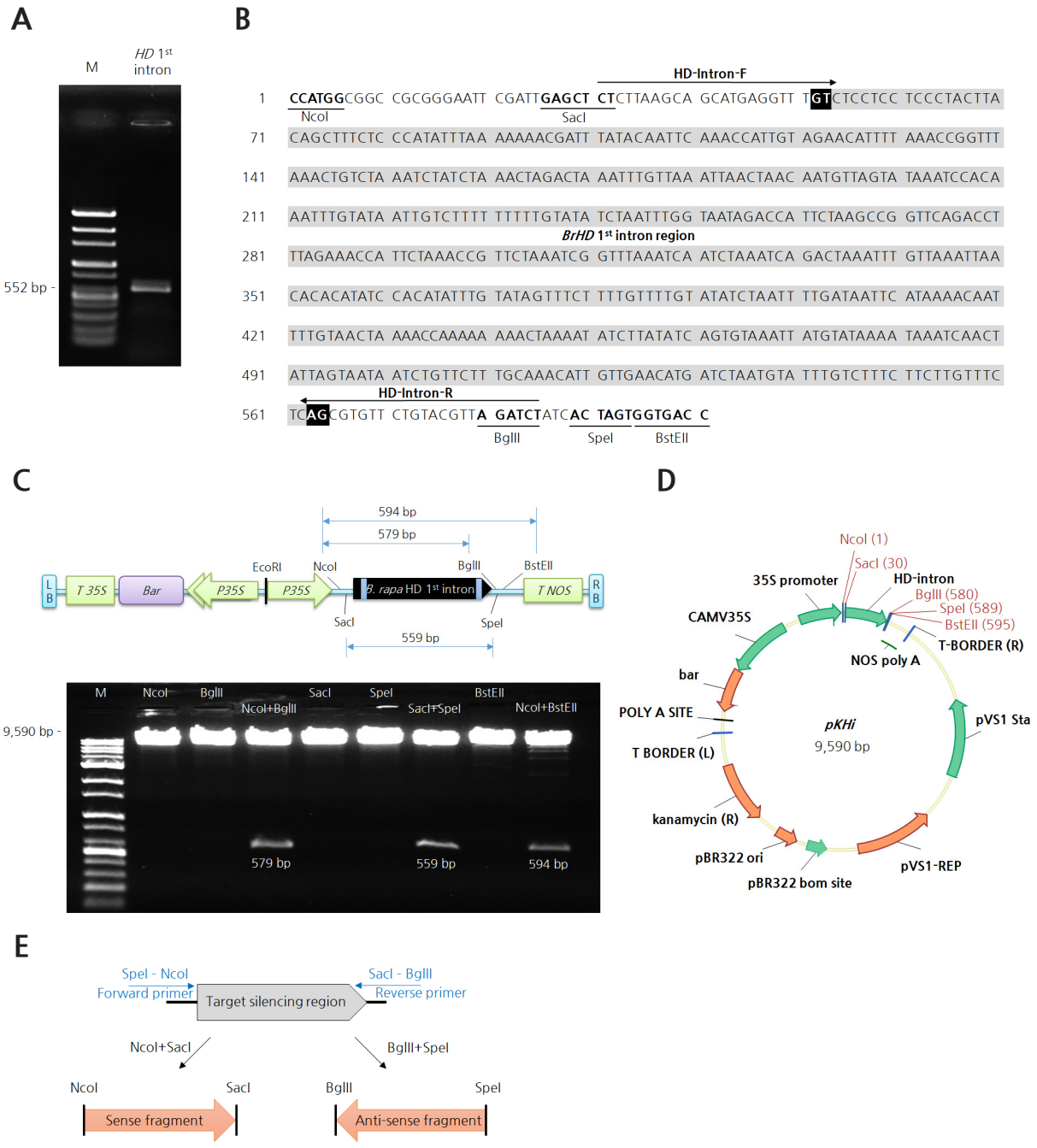

Fig. 1. Construction of the *pKHi* vector system. (A) The *pKHi* vector system was constructed by introducing the 552-bp *BrHD* first intron region. **(B)** GT-AG splicing signals in *BrHD*, black boxes. **(C)** The intron is flanked by unique NcoI, SacI, BglII, SpeI, and BstEII enzyme sites. **(D)** The constructed *pKHi* vector was verified by restriction enzyme mapping. **(E)** To construct the ishpRNA using PCR, SpeI-NcoI and SacI-BglII sites were added to the 5′ and 3′ ends of the amplified target silencing region.

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

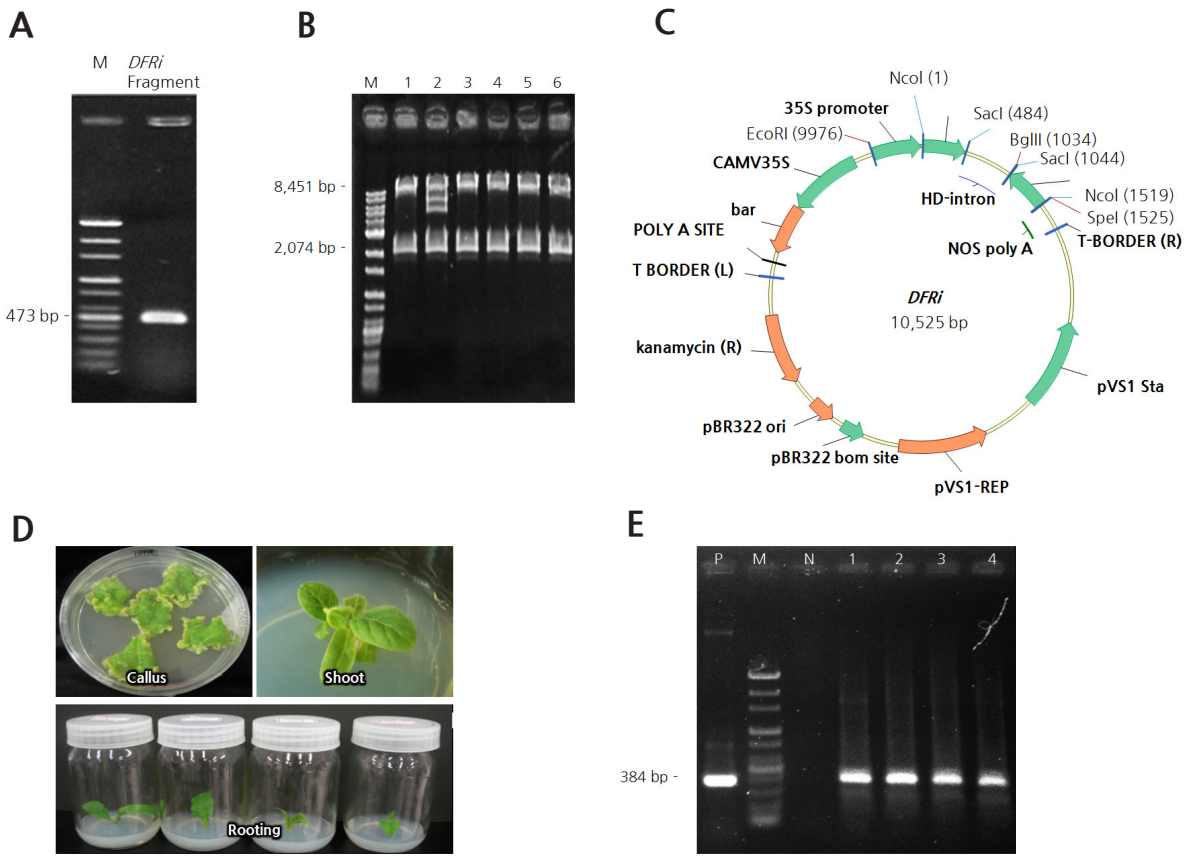

Fig. 2. Construction of *DFR* gene-silencing (*DFRi*) construct and generation of transgenic tobacco using the *pKHi* vector system. **(A)** RT-PCR product used to generate hpRNA for *DFR* siRNA. M, 100-bp DNA ladder. **(B)** The DFR hpRNA fragment was cloned into the *pKHi* vector, and individual clones were analyzed for the insertion by EcoRI-SpeI digestion. M, 100-bp DNA ladder; 1 - 6, *DFRi* vector plasmid. **(C)** Construction of the *DFRi* vector. T-BORDER (L), left border; *POLY A SITE*, *CaMV* 35S terminator; *Bar*, *bialaphos resistance* gene; *CAMV*35S, duplicated *CaMV* 35S promoter; 35S promoter, *CaMV* 35S promoter; DFR sense, sense direction of *DFR* siRNA fragment; *HD* intron, *BrHD* first intron region; *DFR* anti, antisense direction of *DFR* siRNA fragment; *NOS* polyA, *nopaline synthase terminator*; T-BORDER (R), right border; *pVS1 Sta*, stability (STA) region from *pVS1* plasmid; *pVS1-REP*, replication origin from *pVS1*; *pBR322* bom site, basis of mobility (bom) site from *pBR322*; *pBR322 ori*, *pBR322* origin of replication; kanamycin (R), *aadA* (kanamycin resistance gene) amplified from *pIG121Hm*. **(D)** *Agrobacterium*-mediated transformation of tobacco with *DFRi* vector. **(E)** PCR analysis of transgenic plants, based on *Bar* from of the *DFRi* vector. P, *DFRi* vector plasmid; M, 100 - bp DNA ladder; N, non-transgenic tobacco; *DFRi*1 - 4, transgenic tobacco lines.

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 ishpRNA 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.

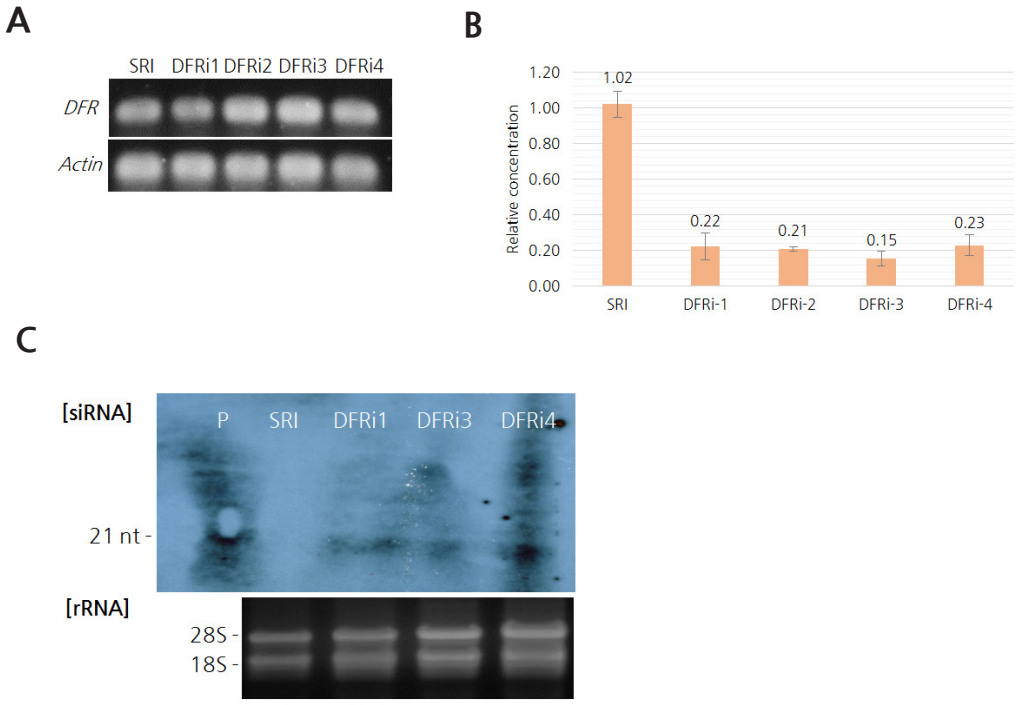

Fig. 3. *DFRi* effectively silences *DFR* in transgenic tobacco. (A) RT-PCR confirmation of *DFR* expression in wild-type and transgenic tobacco plants. SRI, non-transgenic tobacco; DFRi1-4, transgenic tobacco lines. **(B)** Analysis of *BrDFR* gene expression by RT-qPCR analysis. Error bars indicate standard deviation. **(C)** Accumulation of the *DFR* siRNA in selected transformants and in non-transgenic tobacco. P, ssRNA marker (21-nt).

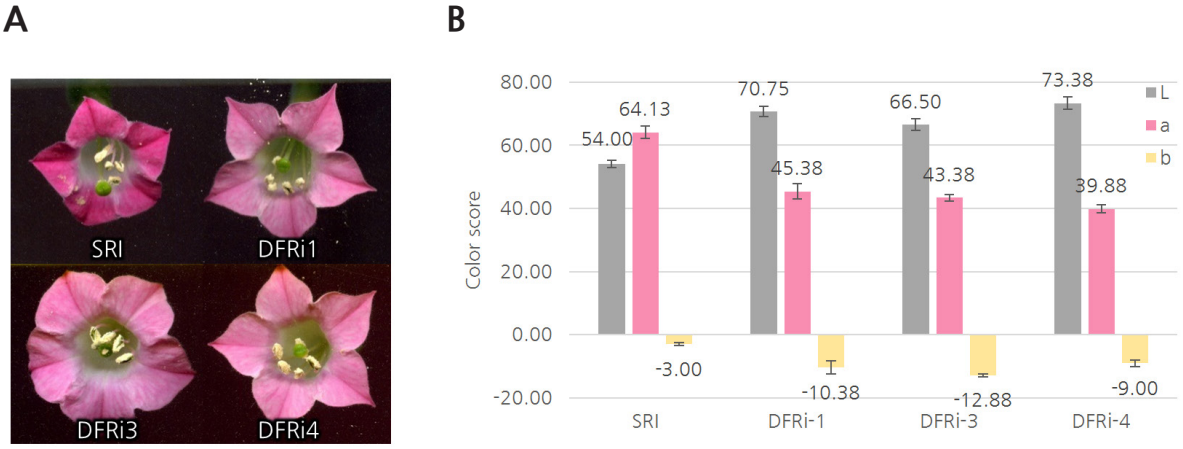

Fig. 4. Anthocyanins in flowers are reduced in *DFRi* transgenic tobacco plants. Flower phenotypes of *DFR* gene-silenced and non-transgenic tobacco plants. **(A)** Phenotype analysis. **(B)** Distribution of petals color based on trivariate (*L**, *a**, and *b**) CIE-Lab color space.

Literature Cited

3,000 rice genomes project (2014) The 3,000 rice genomes project. Gigascience 3:7. doi:10.1186/2047-217X-3-7

Abdurakhmonov IY, Ayubov MS, Ubaydullaeva KA, Buriev ZT, Shermatov SE, Ruziboev HS, Shapulatov UM, Saha S, Ulloa M, et al (2016) RNA interference for functional genomics and improvement of cotton (*Gossypium* sp.). Front Plant Sci 7:202. doi:10.3389/fpls.2016.00202

***Arabidopsis* Genome Initiative** (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 408:796-815. doi:10.1038/35048692

Aufsatz W, Mette MF, van der Winden J, Matzke AJ, Matzke M (2002) RNA-directed DNA methylation in *Arabidopsis*. Proc Natl Acad Sci USA 99:16499-16506. doi:10.1073/pnas.162371499

Childers CC, Brecht JK (1996) Colored sticky traps for monitoring *Frankliniella bispinosa* (Morgan)(Thysanoptera: Thripidae) during flowering cycles in citrus. J Econ Entomol 89:1240-1249. doi:10.1093/jee/89.5.1240

Clemente TE, Cahoon EB (2009) Soybean oil: genetic approaches for modification of functionality and total content. Plant Physiol 151:1030-1040. doi:10.1104/pp.109.146282

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19:11-15

Gan X, Stegle O, Behr J, Steffen JG, Drewe P, Hildebrand KL, Lyngsoe R, Schultheiss SJ, Osborne EJ, et al (2011) Multiple reference genomes and transcriptomes for *Arabidopsis thaliana*. Nature 477:419-423. doi:10.1038/nature10414

Hajdukiewicz P, Svab Z, Maliga P (1994) The small, versatile pPZP family of *Agrobacterium* binary vectors for plant transformation. Plant Mol Biol 25:989-994. doi:10.1007/BF00014672

Haun W, Coffman A, Clasen BM, Demorest ZL, Lowy A, Ray E, Retterath A, Stoddard T, Juillerat A, et al (2014) Improved soybean oil quality by targeted mutagenesis of the *fatty acid desaturase 2* gene family. Plant Biotechnol J 12:934-940. doi:10.1111/pbi.12201

He Q, Shen Y, Wang M, Huang M, Yang R, Zhu S, Wang L, Xu Y, Wu R (2011) Natural variation in petal color in *Lycoris longituba* revealed by anthocyanin components. PLoS ONE 6:e22098. doi:10.1371/journal.pone.0022098

Heilersig HJ, Loonen A, Bergervoet M, Wolters AM, Visser RG (2006) Post-transcriptional gene silencing of GBSSI in potato: effects of size and sequence of the inverted repeats. Plant Mol Biol 60:647-662. doi:10.1007/s11103-005-5280-6

Helliwell C, Waterhouse P (2003) Constructs and methods for high-throughput gene silencing in plants. Methods 30: 289-295. doi:10.1016/S1046-2023(03)00036-7

Helliwell CA, Wesley SV, Wielopolska AJ, Waterhouse PM (2002) High-throughput vectors for efficient gene silencing in plants. Funct Plant Biol 29:1217-1225. doi:10.1071/FP02033

Hirai S, Kodama H (2008) RNAi vectors for manipulation of gene expression in higher plants. Open Plant Sci J 2:21-30. doi:10.2174/1874294700802010021

Holton TA, Cornish EC (1995) Genetics and biochemistry of anthocyanin biosynthesis. Plant Cell 7:1071-1083. doi:10.1105/tpc.7.7.1071

Jyotishwaran G, Kotresha D, Selvaraj T, Srideshikan SM, Rajvanshi PK, Jayabaskaran C (2007) A modified freeze-thaw method for efficient transformation of *Agrobacterium tumefaciens*. Curr Sci 93:770-772

Kumar R, Vashisth D, Misra A, Akhtar MQ, Jalil SU, Shanker K, Gupta MM, Rout PK, Gupta AK, Shasany AK (2016) RNAi down-regulation of *cinnamate-4-hydroxylase* increases artemisinin biosynthesis in *Artemisia annua*. Sci Rep 6:26458. doi:10.1038/srep26458

Kim SB, Yu JG, Lee GH, Park YD (2012) Characterization of *Brassica rapa* S-adenosyl-L-methionine synthetase gene including its roles in biosynthesis pathway. Hortic Environ Biotechnol 53:57-65. doi:10.1007/s13580-012-0084-5

Lee GH, Kang YJ, Yi SK, Lim SB, Park YD (2010) Development of a highly effective T-DNA inserted mutant screening method in a Chinese cabbage (*Brassica rapa* L. spp. *pekinensis*) reverse genetics system. Plant Biotechnol Rpt 4:201-211. doi:10.1007/s11816-010-0137-0

Lee WS, You JA, Chung H, Lee YH, Baek NI, Yoo JS, Park YD (2008) Molecular cloning and biochemical analysis of Dihydroflavonol 4-Reductase (*DFR*) from *Brassica rapa* ssp. *pekinesis* (Chinese cabbage) using a heterologous system. J Plant Biol 51:42-47. doi:10.1007/bf03030739

Liu Q, Singh SP, Green AG (2002) High-stearic and High-oleic cottonseed oils produced by hairpin RNA-mediated post-transcriptional gene silencing. Plant Physiol 129:1732-1743. doi:10.1104/pp.001933

Mo R, Zhang N, Yang S, Zhang Q, Luo Z (2016) Development of a transient ihpRNA-induced gene silencing system for functional analysis in Persimmon (*Diospyros kaki* Thunb.) Korean J Hortic Sci Technol 34:314-323. doi:10.12972/kjhst.20160032

Niu SS, Xu CJ, Zhang WS, Zhang B, Li X, Lin-Wang K, Ferguson IB, Allan AC, Chen KS (2010) Coordinated regulation of anthocyanin biosynthesis in Chinese bayberry (*Myrica rubra*) fruit by a R2R3 MYB transcription factor. Planta 231:887-899. doi:10.1007/s00425-009-1095-z

Pall GS, Hamilton AJ (2008) Improved northern blot method for enhanced detection of small RNA. Nat Protoc 3:1077-1084. doi:10.1038/nprot.2008.67

Park JH, Kim HS, Lee GH, Yu JG, Park YD (2016) Stable inheritance of an integrated transgene and its expression in phenylethylisothiocyanate-enriched transgenic Chinese cabbage. Korean J Hortic Sci Technol 34:112-121. doi:10.12972/kjhst.20160013

Park JS, Yu JG, Park YD (2017) Characterization of a drought tolerance-related gene of Chinese cabbage in a transgenic tobacco plant. Hortic Environ Biotechnol 58:48-55. doi:10.1007/s13580-017-0157-6

Schmitzer V, Veberic R, Osterc G, Stampar F (2010) Color and phenolic content changes during flower development in groundcover rose. J Am Soc Hortic Sci 135:195-202

Siu FK, Lee LT, Chow BK (2008) Southwestern blotting in investigating transcriptional regulation. Nat Protoc 3:51-58. doi:10.1038/nprot.2007.492

Smith NA, Singh SP, Wang MB, Stoutjesdijk PA, Green AG, Waterhouse PM (2000) Total silencing by intron-spliced hairpin RNAs. Nature 407:319-320. doi:10.1038/35030305

Song X, Cao X (2016) Small RNA extraction and detection in rice (*Oryza sativa*). Curr Protoc Plant Biol 1:79-87. doi:10.1002/cppb.20005

Tan J, Wang M, Tu L, Nie Y, Lin Y, Zhang X (2013) The flavonoid pathway regulates the petal colors of cotton flower. PLoS One 8:e72364. doi: 10.1371/journal.pone.0072364

Wang MB, Waterhouse PM (2000) High-efficiency silencing of a *beta-glucuronidase* gene in rice is correlated with repetitive transgene structure but is independent of DNA methylation. Plant Mol Biol 43:67-82. doi:10.1023/A:1006490331303

Wang X, Wang H, Wang J, Sun R, Wu J, Liu S, Bai Y, Mun JH, Bancroft I, et al (2011) The genome of the mesopolyploid crop species *Brassica rapa*. Nat Genet 43:1035-1039. doi:10.1038/ng.919

Wassenegger M (2000) RNA-directed DNA methylation. Plant Mol Biol 43:203-220. doi:10.1007/978-94-011-4183-3_6

Wesley SV, Helliwell CA, Smith NA, Wang MB, Rouse DT, Liu Q, Gooding PS, Singh SP, Abbott D, et al (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. Plant J 27:581-590. doi:10.1046/j.1365-313X.2001.01105.x

Xia Z, Zhao Z, Chen L, Li M, Zhou T, Deng C, Zhou Q, Fan Z (2016) Synergistic infection of two viruses MCMV and SCMV increases the accumulations of both MCMV and MCMV-derived siRNAs in maize. Sci Rep 6:20520. doi:10.1038/srep20520

Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL (2012) Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics 13:134. doi:10.1186/1471-2105-13-134

Ye R, Chen Z, Lian B, Rowley MJ, Xia N, Chai J, Li Y, He XJ, Wierzbicki AT, Qi Y (2016) A dicer-independent route for biogenesis of siRNAs that direct DNA methylation in *Arabidopsis*. Mol Cell 61:222-235. doi:10.1016/j.molcel.2015.11.015