A Thesis for the Degree of Master of Science

Visual monitoring system for virus-induced gene silencing (VIGS) in pepper fruit

고추 과실에서의 유전자 기능 분석을 위한
시각적 **VIGS** 방법

August, 2017

JIHYUN KIM

MAJOR IN HORTICULTURAL BIOTECHNOLOGY
DEPARTMENT OF PLANT SCIENCE
THE GRADUATE SCHOOL OF
SEOUL NATIONAL UNIVERSITY

Visual monitoring system for virus-induced gene silencing (VIGS) in pepper fruit

UNDER THE DIRECTION OF DR. DOIL CHOI
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
SEOUL NATIONAL UNIVERSITY

BY
JIHYUN KIM

MAJOR IN HORTICULTURAL BIOTECHNOLOGY
DEPARTMENT OF PLANT SCIENCE
THE GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY

August, 2017

APPROVED AS A QUALIFIED THESIS OF JIHYUN KIM
FOR THE DEGREE OF MASTER OF SCIENCE
BY THE COMMITTEE MEMBERS

CHAIRMAN Byoung-Cheorl Kang, Ph.D.

VICE-CHAIRMAN Doil Choi, Ph.D.

MEMBER Cecile Segonzac, Ph.D.

Visual monitoring system for virus-induced gene silencing (VIGS) in pepper fruit

JIHYUN KIM

MAJOR IN HORTICULTURAL BIOTECHNOLOGY
DEPARTMENT OF PLANT SCIENCE
THE GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY

ABSTRACT

After completion of sequencing multiple plant genomes, virus-induced gene silencing (VIGS) has become an option of functional genomics tool for specific plants, which are recalcitrant to *Agrobacterium*-mediated transformation such as pepper. Although VIGS in plants has being widely employed as a powerful tool for functional genomics, scattering phenotypic effects resulted by irregular gene silencing become a main problem to be overcome especially in fruit tissue. I improved VIGS system based on tobacco rattle virus (TRV) by using *An2* MYB transcription factor, which is the genetic determinant of purple color by accumulation of anthocyanin in pepper fruit. Silencing of endogenous *An2* in the anthocyanin-rich pepper with modified TRV vector for ligation-independent cloning (LIC) resulted in deficiency of the purple pigment in leaves, flowers and fruits. Infection with TRV-LIC including a tandem construct of *An2* and *Phytoene*

desaturase (*PDS*) resulted in a typical photobleaching in leaves without the purple pigment whereas the silencing of *PDS* alone lead to photobleached and purple-colored leaves. Plants infected with TRV-LIC vector including fragments of both *An2* and pepper *Golden-like 2* (*CaGLK2*) showed color change from green to pale green in fruits. Co-silencing of *Capsaicin synthase* with *An2* in fruits resulted in decreased level of capsaicin and dihydrocapsaicin accumulation measured by HPLC analysis coupled with absence of the purple pigment in fruits. These results imply that VIGS with tandem constructs anchoring *An2* as a visible reporter can accelerate a study about functions of metabolic genes in fruit development.

Key words: Virus-Induced Gene Silencing (VIGS), Pepper fruit, *Tobacco rattle virus* (TRV), pepper *Golden-like 2* (*CaGLK2*), *Capsaicin synthase* (*CS*), *Anthocyanin2* (*An2*), Reporter system, anthocyanin, capsaicinoid

Student number: 2013-21104

CONTENTS

ABSTRACT ····· i

CONTENTS ····· iii

LIST OF TABLE ····· v

LIST OF FIGURES ····· vi

LIST OF ABBREVIATIONS ····· vii

INTRODUCTION ····· 1

LITERATURE REVIEWS ····· 4

Virus-induced gene silencing (VIGS) ····· 4

VIGS methods in pepper ····· 5

Reporter system of VIGS in Solanaceae plants ····· 7

MATERIALS AND METHODS ····· 8

Plant materials and growing conditions ····· 8

Plasmid construction ····· 8

Agro-infiltration ····· 11

RNA isolation and quantitative RT-PCR analysis ····· 11

Capsaicinoid extraction and HPLC analysis ·····12

RESULTS ····· 14

Optimization of VIGS system for gene function study in pepper fruits ····· 14

Validation of endogenous *An2* as a reporter gene in different tissues ····· 17

Validation of the co-silencing using tandem construct with *PDS* and *An2* ····· 20

Validation of gene silencing using fruit specific *CaGLK2* in pepper fruit ····· 23

Co-silencing of *Capsaicin synthase* and *An2* for capsaicinoid study in pepper ····· 26

DISCUSSION ····· 30

LITERATURE CITED ····· 33

ABSTRACT IN KOREAN ····· 37

LIST OF TABLE

Table 1. List of primer sequences used in this study ····· 10

LIST OF FIGURES

Figure 1. Cloning procedure for construction of TRV2 vectors using reporter gene, *An2* and details of pTRV1 and pTRV2 plasmids 15

Figure 2. Anthocyanin-mediated visualization of VIGS in pepper using TRV2-LIC and *An2* as a reporter 18

Figure 3. Co-silencing of *PDS* and *An2* in pepper 21

Figure 4. Co-silencing of pepper *Golden-like 2* (*CaGLK2*) and *An2* in pepper fruit 24

Figure 5. Co-silencing of *Capsaicin synthase* (*CS*) and *An2* 28

LIST OF ABBREVIATIONS

CaGLK2	Pepper *Golden-like 2*
CS	*Capsaicin synthase*
Del	*Delila*
DPA	Days post-anthesis
dsRNA	Double-stranded RNA
GSP	Gene specific primer
GOI	Gene of interest
HPLC	High performance liquid chromatography
LIC	Ligation independent cloning
NMH	*Capsicum annuum* cv. NuMex Halloween
PDS	*Phytoene desaturase*
PTGS	Post-transcriptional gene silencing
Ros1	*Rosea1*
siRNA	Small-interfering RNA
TRV	*Tobacco rattle virus*
VIGS	Virus-induced gene silencing

INTRODUCTION

The completion of whole genome sequencing in major crops has accelerated functional genomic technologies to reveal genes of unknown function (Burch-Smith et al., 2004; Collins et al., 2003; Fu et al., 2006). Several methods have been invented but a rapid and massive screening tool is required for handling innumerable genes. Subsequently, virus-induced gene silencing (VIGS), one of the most broadly used functional genetic tool, has been developed for Solanaceae plants (Becker and Lange, 2010; Chung et al., 2004; Liu et al., 2002a).

Pepper is nutritionally and economically important vegetable crop, food additive and health product. Pepper become very popular in global market since it can synthesize many health-promoting compounds such as capsaicinoids, vitamin C, and carotenoids (Wahyuni et al., 2013). Thus, breeding and biotechnology to enhance these metabolites are being demanded for improving human health. However, genes regulating the biosynthetic pathways of these compounds are not fully elucidated. The genome sequence of pepper can help to overcome this obstacle and accelerate the identification of many genes potentially associated with important agronomic traits (Kim et al., 2014). Pepper is a close relative of tomato and ?????? can take advantages of tomato, a model plant for fleshy fruit biology and biotechnology. Unlike tomato and other Solanaceae plants, pepper is recalcitrant to *Agrobacterium*-mediated transformation for overexpression or knock-out of gene. Thus, virus-induced gene silencing (VIGS) appears to be a powerful technology for

determining the function of unknown genes by down-regulation of genes in Solanaceae plants including pepper. Although there have been several attempts to improve VIGS system in pepper, previous VIGS systems have several limitations, which restrict functional study of genes involved in biosynthetic pathways of metabolites synthesized in fruits. A VIGS method was optimized for analyzing the function of genes only in vegetative tissues of pepper with *Tobacco rattle virus* (TRV) vector under low temperature (Chung et al., 2004). In order to develop VIGS system for pepper fruit biology, a VIGS tool was used with the *Pepper huasteco yellow veins virus* (PHYVV) vector causing severe viral symptom, which was inappropriate to interpret precise phenotypes by silencing of a target gene. Another experiment was also devised for analysis of genes expressed in the late stage of fruit development but not in a whole fruit. (del Rosario Abraham-Juárez et al., 2008; Tian et al., 2014). Although VIGS has been widely employed for functional genomics of fruits in other plants, it has a predominant problem to hinder functional study of gene with scattering phenotypic effects by uneven gene silencing in fruit tissue. To hurdle the drawback of VIGS, visual reporter systems were developed using *Green fluorescence protein* (*GFP*) (Quadrana et al., 2011) and *Antirrhinum majus Delila* and *Rosea1* transcription factors (*DEL-ROS*) of anthocyanin pathway (Orzaez et al., 2009) under *GFP*-overexpressing and anthocyanin-rich transgenic plants, respectively. Because silencing areas was visually traceable in these systems, dissection and sampling of tissues for subsequent metabolic analysis successfully identified gene functions impacting the relevant metabolites (Fantini et al., 2013; Orzaez et al., 2009; Quadrana et al., 2011). Therefore, it is essential to develop a visually traceable tool to study the function of genes expressed in pepper fruit

including the genes for biosynthesis of secondary metabolites.

In this study, I optimized the VIGS system to detect region of gene silencing without special apparatus on pepper fruit. For this purpose, I adapted TRV-LIC VIGS system (Dong et al., 2007) for high-throughput cloning and used *Anthocyanin 2* (*An2*) (Borovsky et al., 2004) as a reporter. *An2* is the genetic determinant of a purple pigmentation by the accumulation of anthocyanin in pepper. In order to validate the improved VIGS system, *PDS*, *CaGLK2*, and *CS* were chosen. *Phytoene desaturase* (*PDS*) involves in carotenoid biosynthetic pathway to control the level of carotenoids. *PDS* is used as a positive control for VIGS since it presents photobleaching in silenced region. Pepper *Golden-like 2* (*CaGLK2*) is expressed in pepper fruit developmental stage and controls chloroplasts development including chlorophyll. *Capsaicin synthase* (*CS*) works in the last step of capsaicinoid biosynthesis pathway and synthesizes capsaicinoids in placenta of pepper fruit. This VIGS system was successfully examined for co-silencing of *PDS*, *CaGLK2* and *CS* coupled with *An2*, and followed by monitoring of phenotype and metabolite accumulation changes in leaves and fruits. These results will advance reverse genetics for fruit traits and ultimately help to understand the molecular functions of novel genes and regulator involved in the biosynthesis of secondary metabolites in pepper fruits.

LITERATURE REVIEW

Virus Induced gene silencing (VIGS)

Virus-induced gene silencing (VIGS) is a useful reverse-genetics method for functional characterization of genes in plants. When virus infects in plants, it starts to replicate viral double-stranded RNA (dsRNA). Dicer, which is an RNase III endonuclease, recognizes and cleaves the dsRNA into small-interfering RNA (siRNA). Argonaute in RNA-induced silencing complex (RISC) matches the sequence of siRNA with that of the single-stranded RNA such as virus or mRNA of plant, subsequently leading down-regulation of the gene expression. These series of processes are called post-transcriptional gene silencing (PTGS). In VIGS system, engineered virus vector that contains a fragment of a plant gene of interest (GOI) can be used to cause the silence of the plant's endogenous targeted gene (Baulcombe, 1999; Dinesh-Kumar et al., 2003; Fu et al., 2006; Hammond et al., 2001). VIGS has several advantages over *Agrobacterium*-mediated transformation methods for study of functional genetics. First of all, it is a transient method that can quickly observe GOI-silenced phenotype in the same generation. Second, it can be used to essential genes involved in reproduction. Last, it works in a variety of crops which have low efficiency of transformation with simple process using cloning and *Agrobacterium*-infiltration (Burch-Smith et al., 2004; Dinesh-Kumar et al., 2003).

VIGS methods in pepper

Pepper is one of important fruit crops with diverse usages for producing healthy metabolites such as capsaicinoids, vitamins, and carotenoids. VIGS is a powerful tool for reverse genetics since pepper is recalcitrant to *Agrobacterium*-mediated transformation in contrast with other model plant like tomato. There have been several attempts for functional analysis of genes in pepper fruits using VIGS. Nevertheless, the previous studies have some limitation, which cannot apply in a method to study the function of genes involved in biosynthetic pathway of health-promoting metabolites. Chung *et al*. (2004) used TRV which has mild symptom as vector. TRV virus is a bipartite RNA virus. In order to employ TRV as a vector for VIGS system, pTRV1 and pTRV2 were modified containing important parts such as movement protein (MP), coat protein (CP), and RNA-dependent RNA polymerase (RdRp) for systemically spreading in plant (Liu et al., 2002b). When *Agrobacterium* cultures carrying pTRV1 and pTRV2 respectively is infiltrated in plant, pTRV1 and pTRV2 expresses in plant cell and silencing of gene is successfully triggered by PTGS in plant. Thus, *Agrobacterium* carrying pTRV1 and pTRV2 with target gene respectively were infiltrated in cotyledons of pepper at 16 ℃ for 1 day with treatment of dark. However, this study was limited on silencing in vegetative tissues. In 2007, to characterize gene related to capsaicinoid biosynthesis pathway, *Comt*, *pAmt*, and *Kas* genes were silenced in pepper fruit using *Pepper huasteco yellow veins virus* (PHYVV) vector (del Rosario Abraham-Juárez et al., 2008). Although it showed the decrease of capsaicinoid content in pepper fruits, plants had strong viral symptom of PHYVV. In order to devise VIGS system for analyzing the function of

genes expressed in pepper fruit, Tian et al. (2014) directly silenced genes related to capsanthin biosynthetic pathway in detached fruits. Capsanthin synthesis occurs in late stage of fruit development. Thus, this VIGS system cannot apply in entire stage of pepper fruit

Reporter system of VIGS in Solanaceae plants

VIGS is a convenient tool to analyze the function of gene in Solanaceae plants. However, the uneven gene silencing is a significant problem that hinders the exact identification and quantification of nonvisual phenotype. To overcome this limitation in fruits especially, there are many attempts for visual reporter systems to trace the effect of VIGS. Previous studies used a *Del*/*Ros1*-overexpressing transgenic tomato line, *Antirrhinum majus Delila* and *Rosea1* transcription factors, presenting an anthocyanin-rich tomato and TRV vector containing a fragment of *Rosea1* and *Delila* for VIGS. The results of experiments contributed to monitor the effect of gene silencing on fruit through purple-faded sector in fruit and to collect the target gene-silenced region, which helped to analyze the definite decrease of metabolites level by down-regulation of the target gene (Fantini et al., 2013; Orzaez et al., 2009). Quadrana et al. (2011) devised a reporter system exploiting transgenic plants based on ectopic expression of *Green fluorescence protein* (*GFP*). The tissues decreased fluorescence under UV was easily sampled and this reporter system offered the high efficiency of target gene-silencing in Arabidopsis leaves and tomato fruits.

MATERIALS AND METHODS

Plant materials and growing conditions

Capsicum annuum cv. NuMex Halloween (hereafter NMH), kindly provided by Prof. Byoung-Cheorl Kang (Seoul National University), was used in this study and maintained in growth chambers. Pungent NMH contained visible amounts of anthocyanin in leaf, stem, flower, and fruit. The plant height was approximately 30 cm. After germination on wet paper towel in plates at 30 °C, the seedlings were transferred to plug trays at 25 °C with a 16/8 h light/dark photoperiod until cotyledons were fully expanded (approximately 2 weeks after germination). After agro-infiltration to the abaxial side of the cotyledons, pepper plants were incubated at 16 °C under dark conditions for 1 day. Four-week-post-infiltrated plants were transplanted to large pots (130/115 mm) and grown at 20 °C with a 16/8 h light/dark photoperiod. The plants were fertilized by WUXAL according to the manufacturer's instructions (WUXAL calcium, AGLUKON, Germany) once every 2 months.

Plasmid construction

The TRV vectors, pTRV1 (Liu et al., 2002b) and pTRV2-LIC (Dong et al., 2007), were kindly provided by Dr. Dinesh Kumar, at UC Davis. The ligation independent cloning (LIC) was conducted as described in Dong et al. and **Figure 1.**

For gene cloning in pTRV2-LIC, GOI was amplified with primers: 5′-CGACGACAAGACCCT (LIC vector adaptor)-gene specific sequences-3′ and 5′-CTTTGTCTAGTG (*An2* adaptor)-gene specific sequences-3′ using SolgTM *Pfu*-X DNA polymerase (Solgent, Korea). *An2* was amplified with primers An2_F and An2_lic_R **(Table 1).** Fragments of *PDS* (CA03g36860, 173 bp), *CS* (CA02g19260, 181 bp), *CaGLK2* (CA10g02900, 222bp), and *An2* (CA10g11650, 258 bp) were amplified from the pepper leaf, placenta, and pericarp cDNA, respectively. TRV2-*GFP* was previously used for VIGS as a control (Sarowar et al., 2007). The insert size of each target gene and *An2* was 150–300 bp and the insert size of fused cDNAs was 400–600 bp in the TRV2-LIC vector. Using the amplified cDNAs of the target genes and *An2* as template, PCR was performed to fuse both cDNA fragments in the sense orientation. The PCR products were purified with DNA Clean and Concentrator™ (Zymo Research, USA). A total 100 ng of purified PCR product was treated with T4 DNA polymerase (New England Biolabs, USA) in 1× reaction buffer containing 10 mM dATP and dithiothreitol at 22 °C for 30 min followed by 20 min of inactivation of T4 DNA polymerase at 70 °C. The TRV2-LIC vector was digested by the restriction enzyme *Pst*I and treated with T4 DNA polymerase and dTTP instead of dATP. A total of 50 ng of PCR product and TRV2-LIC vector were mixed and incubated at 65 °C for 1 min and then 22 °C for 10 min. The mixture was transformed into *E. coli* DH10B or DH5α competent cells. Transformants were selected by PCR using primers for sequencing **(Table 1)** and confirmed by DNA sequencing. The plasmids from the transformants were introduced into *Agrobacterium tumefaciens* strain GV3101 using the freeze–thaw method (Chen et al., 1994).

Table 1. List of primer sequences used in this study

Primer	Sequence (5'-3')
Construct	
GLK2_lic_F	CGACGACAAGACCCT TGTTGTATCTACACCATTGAGC
GLK2_An2_R	CTTTGTCTAGTG TTTTCCTTTGACTTTGTTTTCACA
CS_lic_F	CGACGACAAGACCCT GAGAAGGGAAACTGCCATTTGA
CS_An2_R	CTTTGTCTAGTG CCTTGCCCAGCTTTGTAATCTT
GFP_lic_F	CGACGACAAGACCCT CACGGCAGACAAACAAAAGA
GFP_An2_R	CTTTGTCTAGTG AAAGGGCAGATTGTGTGGAC
An2_F	CACTAGACAAAGACGAACGCGAC
An2_lic_R	GAGGAGAAGAGCCCT CAGAAAAGTCATCCCAACCATCAC
PDS_lic_F	CGACGACAAGACCCT CTGGTGACTACACGAAACAG
PDS_An2_R	CTTTGTCTAGTG ACTATGCTAACTACGCTTGC
Sequencing	
TRV2_seq_F	CTGTTTGAGGGAAAAGTAG
TRV2_seq_R	CAAAAGACTTACCGATCAATC
qRT-PCR	
GLK2_F	CTTTCCTTCACCAACGAGATTTG
GLK2_R	CTATAGGAAGTGGCGTTTGTGGCTTTG
CS_F	TTCCCATATAGCCCACTTGC
CS_R	ACTACAAGCAAATTACCACCTTC
PDS_F	AGCAAAGCAAAAATATTGAAGTA
PDS_R	GCTTTCCTGATAAGACAGC
An2_F	CTGATATTGACCTATGGAATCTACTTAATTAGTTTCA
An2_R	CCCTACTTGTACTTGGGATAGTACGAACAG
CaActin_F	CCACCTCTTCACTCTCTGCTCT
CaActin_R	ACTAGGAAAAACAGCCCTTGGT

Agro-infiltration

Agrobacterium tumefaciens strain GV3101 carrying pTRV1, pTRV2::*GFP*, pTRV2-LIC::*GFP*::*An2*, pTRV2-LIC::*PDS*::*An2*, pTRV2-LIC::*CaGLK2*::*An2*, pTRV2-LIC::*CS*::*An2*, and was grown overnight at 28 °C in 10 mL YEP medium containing rifampicin (50 μg/mL) and kanamycin (50 μg/mL) as described (Chung et al., 2004). The transformed *Agrobacterium* was harvested by centrifugation at 13,000×*g* for 15 min at 20 °C, and resuspended in 10 mM MES, 10 mM $MgCl_2$, and 200 μM acetosyringone to a final OD600 nm of 0.7. Cell suspensions were incubated at room temperature with inverting for 4 h. *Agrobacterium* cultures containing pTRV1 and pTRV2-LIC carrying any GOI were mixed at a 1:1 ratio and infiltrated into the abaxial side of both cotyledons. Leaves from 4-week-old plants and placenta from fruits from plants 30 days post-anthesis (DPA) were harvested and immediately frozen in liquid nitrogen for qRT-PCR and HPLC. Due to a high frequency of fruit abscission, at least five silenced plants per each construct were grown at 20 °C until harvest.

RNA isolation and quantitative RT-PCR analysis

The total RNA from 100 mg of tissue was extracted using the TRIzol® reagent (Invitrogen, USA). Total RNA (5 μg) was reverse-transcribed with Oligo (dT) primers and Superscript II (Invitrogen), according to the manufacturer's instructions. Subsequently, qRT-PCR was conducted to analyze gene expression level using the SYBR Green PCR master mix (Invitrogen, USA) and gene-specific primers **(Table 1)**

in the Rotor-Gene 6000 apparatus (QIAGEN, USA), according to the manufacturer's instructions. All statistical analyses were conducted as described in the manufacturer's protocol. To normalize the expression levels, the transcript level of *CaActin* (CA00g80270) was used as a control. Duplicates from 3 biological replicates were used in the qRT-PCR analysis.

Capsaicinoid extraction and HPLC analysis

The placental tissue of each fruit at 30 days-post anthesis was isolated and immediately frozen in liquid nitrogen. For sampling in *An2*-silenced plants, purple pigment-depleted placenta was carefully collected. Previous method of capsaicinoid extraction (Han et al., 2013) was modified for frozen tissues. Approximately 100 mg of frozen powder from pooled samples of two fruits was extracted with 1.5 mL of an ethyl acetate and acetone mixture (6:4) using TissueLyser II (QIAGEN, USA) at room temperature for 10 min and incubated with shaking at 37 °C for 1 h. After centrifugation at 12,000×*g* for 5 min at room temperature, 600 µL of the supernatant was transferred to a new tube and evaporated in an Automatic Environmental SpeedVac System AES1010 (Operon, Korea). The extract was dissolved in 500 µL methanol and filtered using an Acrodisc® LC 13-mm syringe filter with a 0.2-µm PVDF membrane (Sigma-Aldrich, USA). Capsaicinoid analysis was performed using the UltiMate® 3000 HPLC (Dionex, USA) including the Inno C-18 column (4.6 mm × 150 mm, YoungJin Biochrom, Korea). A UV detector was operated at 280 nm and the data acquisition was performed with Chromeleon software. Separation of capsaicinoids was achieved under 75% MeOH at a flow rate of

1 mL/min. Each 10 μL aliquot was analyzed with HPLC. The HPLC analyses were performed at NICEM, Seoul National University. Capsaicin and dihydrocapsaicin used as standard compounds were purchased from Sigma-Aldrich (M2028 and M1022, respectively) (Han et al., 2013).

Results

Optimization of VIGS system for gene function study in pepper fruits

To improve VIGS system, a couple of conditions were determined in terms of appropriate virus vector, temperature, and reporter gene. First, TRV vector was used for stable VIGS reaction since TRV can systemically spread in whole tissue of pepper including reproductive tissue and cause mild viral symptom as well as TRV2-LIC vector is high through-put to cloning (Chung et al., 2004; Dong et al., 2007; Liu et al., 2002b). Second, infiltrated plants were grown at 20℃ to maintain gene-silencing from post-infiltrated seedling to fruiting. The *An2* regulates expression of genes involved in biosynthesis and accumulation of anthocyanin in purple pepper (Borovsky et al., 2004). Third, *Anthocyanin 2* (*An2*), R2R3-MYB transcription factor, was used as a reporter gene in purple pepper and a fragment of *An2* in tandem with GOI was inserted into pTRV2 vector with target gene to trace gene-silencing for several months (Figures 1).

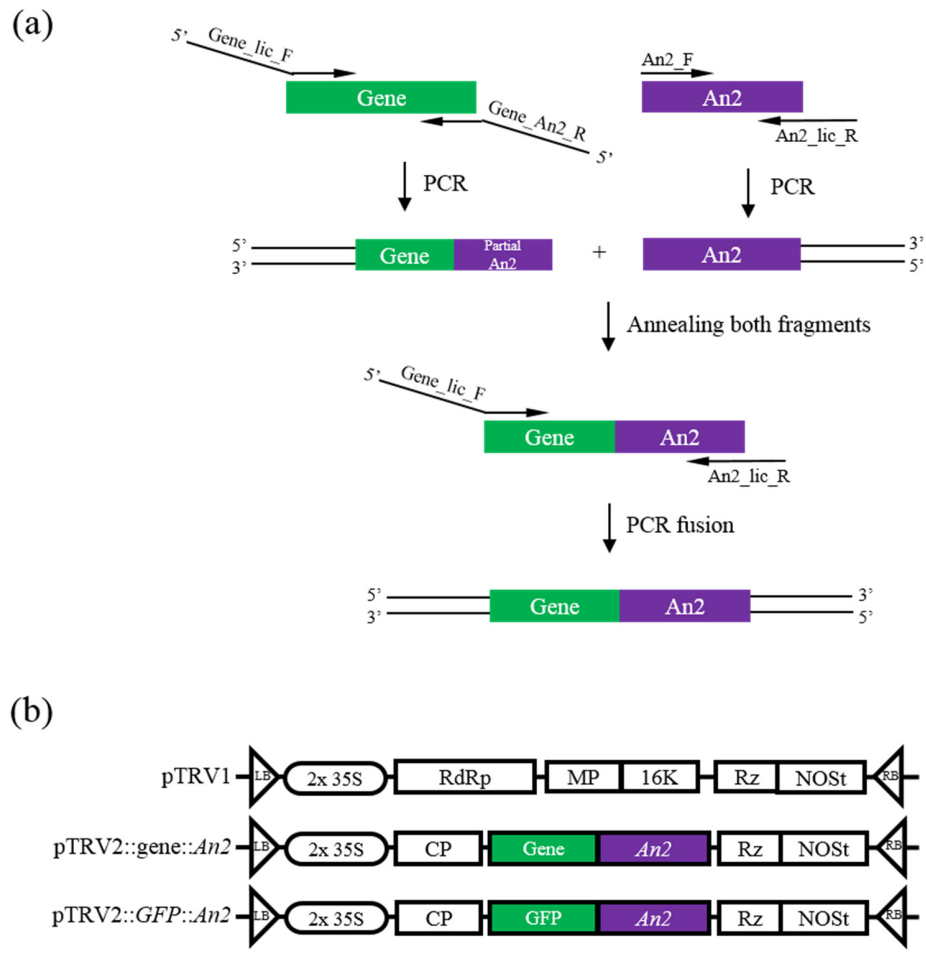


Figure 1. Cloning procedure for construction of TRV2 vectors using reporter gene, *An2* and details of pTRV1 and pTRV2 plasmids

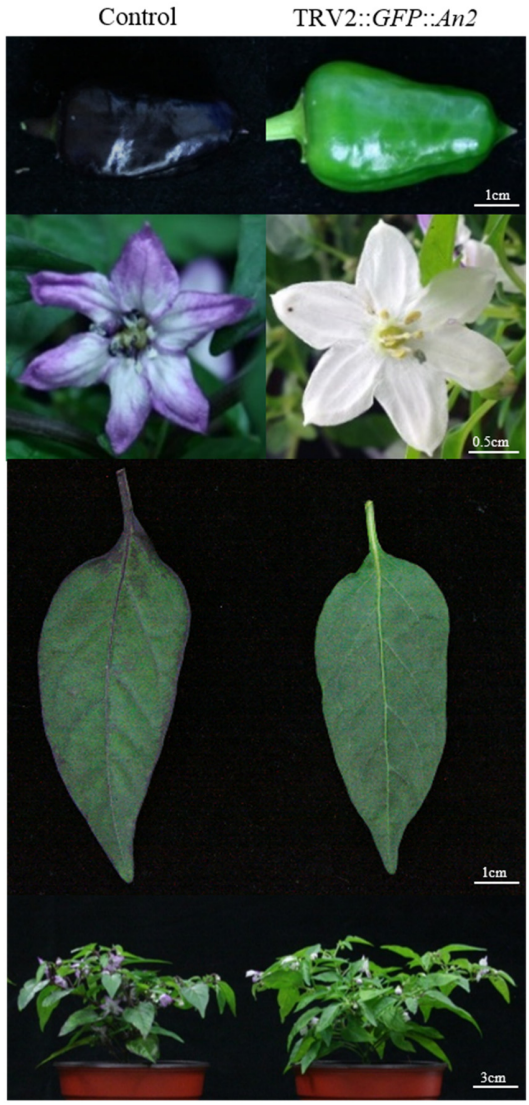
(a) The gene of interest was amplified by PCR using gene specific primers (GSP) with LIC adaptor (Gene_lic_F) and *An2* adaptor (Gene_An2_R) using cDNA. *An2* was amplified using *An2* specific primer with *An2* adaptor (An2_F) and LIC adaptor (An2_lic_R). *An2* adaptor sequence was able to anneal both gene and *An2* fragments and subsequent PCR using LIC adaptor-attached primers was able to fuse both

fragments. (b) Schematic of TRV1 and TRV2 construct: LB, left border; RB, right border; RdRp, RNA-dependant RNA polymerase; MP, movement protein; 16K, 16 Kd protein; Rz, self-cleaving ribozyme; NOSt, NOS terminator; CP, coat protein; MCS, multiple cloning site; Gene, target gene; *GFP*, *green fluorescent protein*; *An2*, *Anthocyanin 2*.

Validation of endogenous *An2* as a reporter gene in different tissues

To confirm suitability of *An2* as a reporter gene for VIGS, gene specific fragment of *An2* (CA10g11650, 258 bp) was integrated with TRV2-LIC vector (Figure 1). TRV2::*GFP* as a control and TRV2::*GFP*::*An2* with TRV1 were agro-infiltrated in both cotyledons of 2-week-old seedlings of NMH to spread out the silencing response into meristematic tissues (Figure 2a). NMH is ornamental plant having purple color in whole organs and pungent taste within fruit. Low temperature enhances silencing of genes in Solanaceae plants from seedlings to fruits (Fu et al., 2006). The infiltrated plants were grown at 20°C to maintain the silencing for long duration and to intensify the silencing efficiency. Compared with the phenotypes of control, *GFP*/*An2*-silenced plant showed systemically uniformed-decrease of anthocyanin pigment in leaf, flower and fruit (Figure 2a). In *An2*-silenced plants, reproductive organs of flower such as stigma and stamen showed absence of the purple pigment. In order to relate the absence of purple pigment in the green fruit with the silencing of *An2*, qRT-PCR was performed to measure the level of gene expression. The amount of *An2* expression was largely decreased in TRV2::*GFP*::*An2* in contrast to control (Figure 2b). Therefore, this VIGS system using TRV2 vector with *An2* reporter gene at 20°C during plant growing is appropriate for studying the function of genes expressed in pepper fruit without severe phenotype effects.

(a)


Control
TRV2::*GFP*::*An2*
1cm
0.5cm
1cm
3cm

(b)

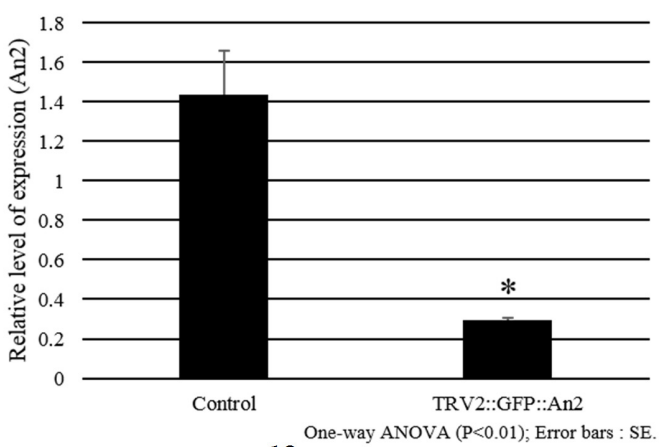

Relative level of expression (An2)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
Control
TRV2::GFP::An2
One-way ANOVA (P<0.01); Error bars : SE.

Figure 2. Anthocyanin-mediated visualization of VIGS in pepper using TRV2-LIC and *An2* as a reporter

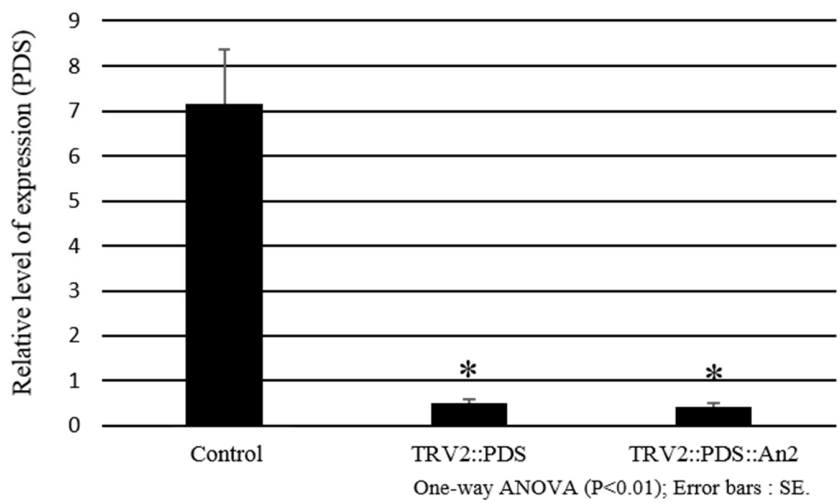
(a) Phenotype from different tissues of TRV2::*GFP* infiltrated pepper (control, left) and TRV2::*GFP*::*An2* infiltrated pepper (right) (b) Expression analysis of *An2* by qRT-PCR in 30 DPA fruits. All the qRT-PCRs were performed using three biological replicates and the data presents the average of three replicates.

Validation of the co-silencing using tandem construct with *PDS* and *An2*

To verify co-silencing of GOI and *An2*, *Phytoene desaturase* (*PDS*) was utilized for co-silencing in this experiment *PDS* silencing leads to photobleaching phenotype. *PDS* involves in carotenoid biosynthetic pathway to control the level chlorophyll, carotenoids, and phytohormones such as abscisic acid and gibberellins. Carotenoids are foremost components of photosynthesis in plants since they contribute to harvest photo-energy and to protect cell from excessive light. Thus, knock-down of *PDS* does not protect plastids within plant cell from light and finally occurs albinotic phenotype called photobleaching (Wang et al., 2009). For this reason, *PDS* was chosen as a positive control of this study. Gene specific fragment of *PDS* (CA03g36860, 173 bp) was tandemly inserted with *An2* within TRV2 vector (Figure 1). *Agrobacterium* carrying TRV2::*PDS* and TRV2::*PDS*::*An2* with TRV1 were infiltrated in cotyledons of NMH. Following observation of phenotypes, qRT-PCR was conducted with 4 months old leaves of each plant to compare the level of gene expression. The result is that *PDS*/*An2*-silenced plants represented deficiency of anthocyanin pigment with photobleaching by co-silencing while *PDS*-silenced plants showed photobleaching only (Figure 3a). In qRT-PCR, the mRNA levels of *An2* was largely decreased only in *PDS/An2*-silenced leaves although there were no substantial differences in the levels of *PDS* expression between *PDS*- and *PDS/An2*-silenced leaves, which implies that silencing of *An2* did not affect *PDS* expression level.

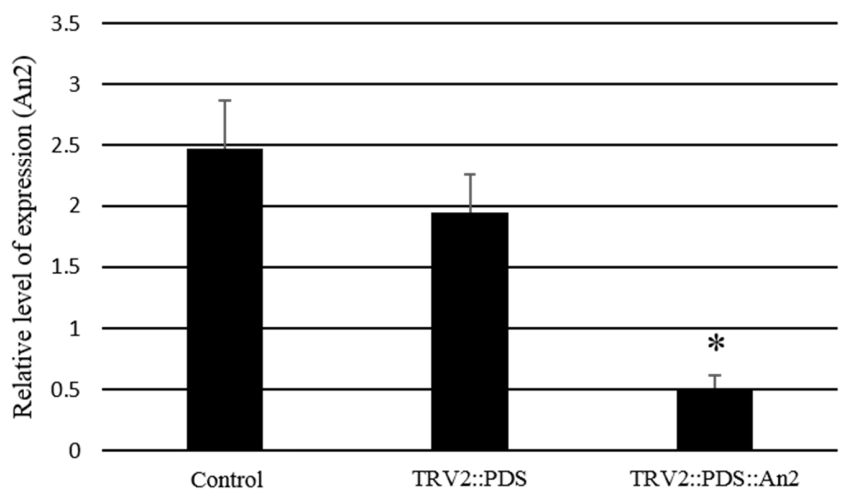
(a)

(b)



One-way ANOVA (P<0.01); Error bars : SE.

(c)



One-way ANOVA (P<0.01); Error bars : SE.

Figure 3. Co-silencing of *PDS* and *An2* in pepper

(a) Two weeks old plants were agro-infiltrated to the abaxial side of the cotyledons with *Agrobacterium* cultures of wild type (control), pTRV1/pTRV2::*PDS*, and pTRV1/pTRV2::*PDS*::*An2*. *PDS*/*An2*-silenced plants resulted in photobleaching and lack of the purple pigment whereas *PDS*-silenced plant showed the purple pigmentation under white leaves in 4 months post-infiltrated plants. (b) In qRT-PCR analysis, *An2* expression level in leaves of *PDS*/*An2*-silenced plants was largely lower than control and *PDS*-silenced plants while (c) *PDS* expression level was highly decreased in *PDS*/*An2*- and *PDS*-silenced plants compared to control. All the qRT-PCRs were performed using three biological replicates and the data presents the average of three replicates.

Validation of gene silencing using fruit specific *CaGLK2* in pepper fruit

To demonstrate efficient silencing of gene specifically expressed in fruit, *CaGLK2* was chosen as a target gene in this study. *CaGLK2*, pepper *Golden-like 2* transcription factor, regulates enzymes involved in the chloroplast development, controlling chlorophyll contents (Brand et al., 2014) and is predominantly expressed in pepper fruits (Kim et al., 2014). The recombinant pTRV2 carrying *CaGLK2* (CA10g02900, 222bp) in tandem with *An2* reporter gene (Figure 1) was introduced into expanded cotyledon and the infiltrated plants grew at 20℃. The phenotype of *GFP*/*An2* and *CaGLK2*/*An2*-silenced plants showed absence of anthocyanin in most tissues while that of wild type plant was purple (data not shown). There was a significant difference in fruit color between *CaGLK2*/*An2*-silenced plants and *GFP*/*An2*-silenced plant presenting pale green and green color, respectively (Figure 4a).

To better understand the correlation between the color variation in fruit and *CaGLK2* transcripts, pericarp tissues of at least six different 30 DPA fruits were collected for further quantification of *CaGLK2* transcripts. In qRT-PCR, level of *CaGLK2* expression significantly decreased in pepper pericarp compared to control (Figure 4b). In Brand et al. (2014), when the expression level of *CaGLK2* decreased in pepper lines and chlorophyll contents was also low, the color of immature pepper fruits becomes pale green. These results imply that the phenotype of pale green fruit color in *CaGLK2*/*An2*-silenced plants can be attributed to the silencing of the *CaGLK2* in fruit.

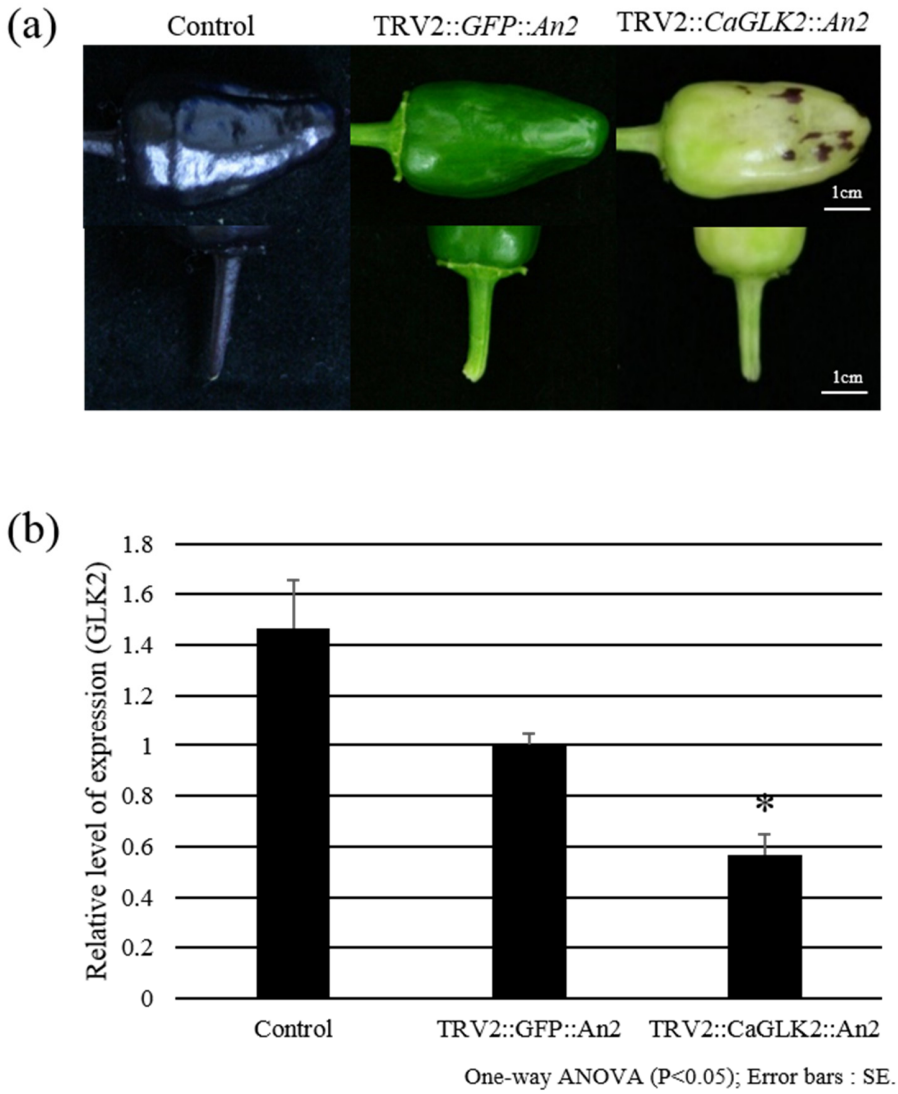


Figure 4. Co-silencing of *Golden-like 2* (*CaGLK2*) and *An2* in pepper fruit

(a) The co-silencing of *CaGLK2* and *An2* resulted in deficiency of anthocyanin pigmentation in pepper pericarp and peduncle (30 DPA) of TRV2::*GFP*::*An2* and TRV2::*CS*::*An2*. (b) Relative expression of *CaGLK2* were significantly lower in the

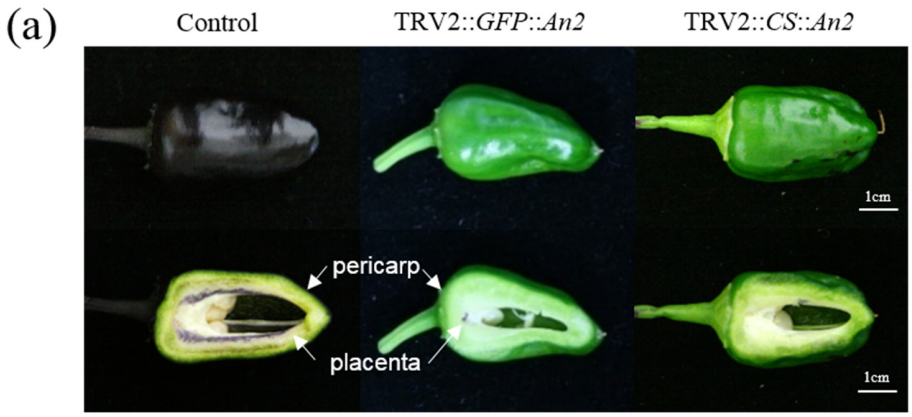
pepper fruits (pericarp) of TRV2::*CaGLK2*::*An2* than in control and TRV2::*GFP*::*An2*. The data represent the mean ± SE (n=6). Asterisk indicates significant differences ($P \leq 0.05$) between TRV2::*GFP*::*An2* and TRV2::*CaGLK2*::*An2*. All the qRT-PCRs were performed using three biological replicates and the data presents the average of three replicates.

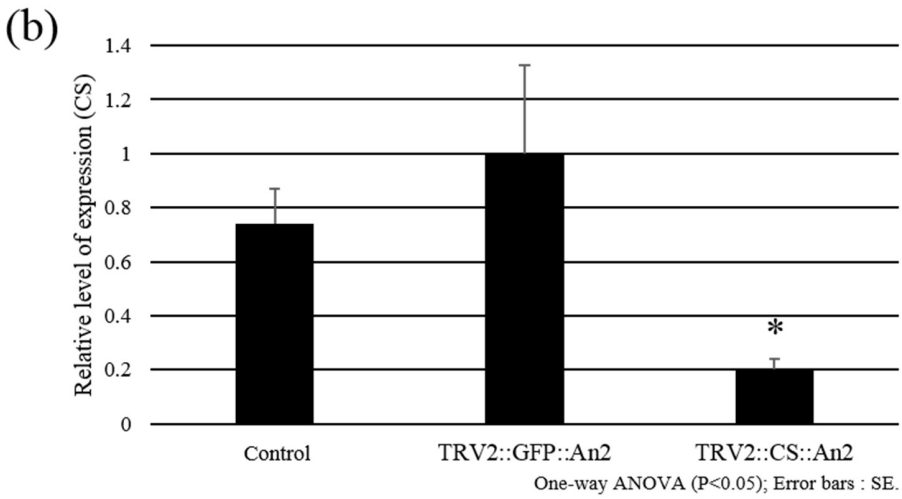
Co-silencing of *Capsaicin synthase* and *An2* for capsaicinoid study in pepper fruit

Capsaicinoids are pungent metabolites and natural vanilloid only found in *Capsicum* species. *Capsaicin synthase* (*CS*), a homologue of acyltransferase, is the genetic factor encoded by *C* or *Pun1* locus controlling the pungency of pepper. *CS* condenses vanillylamine and 8-methyl-6-nonenoyl-CoA synthesized from either valine or leucine, and produces capsaicinoids in the last step of capsaicin synthesis pathway (Mazourek et al., 2009; Stewart et al., 2005). *CS* is only expressed during placenta development in pepper fruit and capsaicinoids are synthesized and accumulated within blister located in placenta surface (Stewart et al., 2005).

To validate co-silencing of *CS* and *An2* in fruits and its potential use as a tool of metabolic genetics, partial cDNA of *CS* (CA02g19260, 181 bp) was inserted within TRV2-LIC tandem system and agro-infiltration was conducted. The infiltrated plants were grown in a growth chamber of 20℃ to maintain silencing reaction. Placenta tissues of at least six different 30 DPA fruits were collected to analyze quantity of capsaicinoids and *CS* transcripts. This time was chosed to take into account the stage-dependent expression of CS and resulting accumulation of capsaicinoid. Compared with the control (wild type) showing primarily purple color due to anthocyanin accumulation, the phenotype of TRV2::*CS*::*An2* and TRV2::*GFP*::*An2* clearly presented green pepper fruit without anthocyanin pigment in pericarp as well as endocarp (Figure 5a). *An2* down-regulation resulted in the loss of anthocyanin pigment in *An2*-silenced fruit previously observed in Figure 2.

To further investigate co-silencing of *CS* and *An2* at the expression level, qRT-PCR were performed with the placenta samples. The transcripts accumulation of *CS* gene reduced compared to control and *GFP*/*An2*-silenced placenta (Figure 5b). To relate whether *CS* silencing affects capsaicinoid accumulation in placenta, capsaicinoids were quantified by HPLC in the same tissues used for qRT-PCR. In nature, the major two analogues accounting for almost 90% of the total capsaicinoids are capsaicin and dihydrocapsaicin (Kozukue et al., 2005). Thus, authentic capsaicin and dihydrocapsaicin were used as internal controls for the quantification of two capsaicinoids. The content of capsaicin and dihydrocapsaicin decreased about 50% compared to *GFP*/*An2*-silenced plant (Figure 5c). No phenotypic change by co-silencing *CS* and *An2* could be observed compared to *An2*-silenced plant suggesting suitability of this VIGS system. Metabolic phenotypes of *CS*-silenced placenta correspond with putative function of *CS*. The anthocyanin accumulation or its depletion in *An2*-silenced placenta had no significant effect on capsaicinoid biosynthesis and *CS* expression.


(a)
Control
TRV2::GFP::An2
TRV2::CS::An2
pericarp
placenta
1cm
1cm


(b)
Relative level of expression (CS)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
Control
TRV2::GFP::An2
TRV2::CS::An2
One-way ANOVA (P<0.05); Error bars : SE.

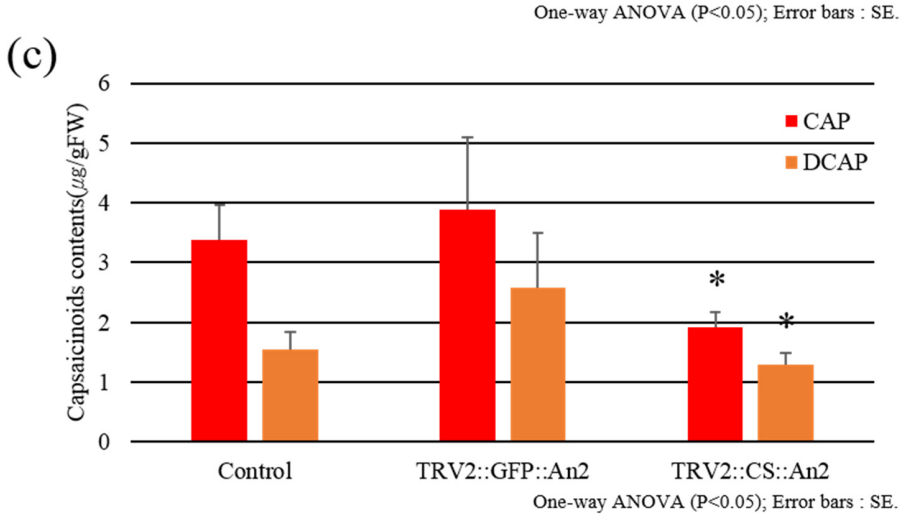

(c)
Capsaicinoids contents(㎍/gFW)
6
5
4
3
2
1
0
CAP
DCAP
*
*
Control
TRV2::GFP::An2
TRV2::CS::An2
One-way ANOVA (P<0.05); Error bars : SE.

Figure 5. Co-silencing of *Capsaicin synthase* (*CS*) and *An2*

(a) Anthocyanin-mediated visualization of co-silencing of *CS* and *An2* in pepper pericarp and placenta (30 DPA). The silencing of *An2* resulted in depletion of anthocyanin pigmentation in pericarp and placenta of TRV2::*GFP*::*An2* and TRV2::*CS*::*An2*. (b) In qRT-PCR, the amount of *CS* transcripts in the co-silenced fruits (placenta) compared to control (wild type) and *An2*-silenced fruits. The expression levels of *CS* significantly more reduced in TRV2::*CS*::*An2* than in control and TRV2::*GFP*::*An2*. (c) In HPLC, Capsaicinoid levels in the co-silenced fruits compared to control and *An2*-silenced fruits. The co-silencing of *An2* and *CS* in placenta resulted in decreased level of capsaicin and dihydrocapsaicin. The data represent the mean ± SE (n=6). Asterisks indicate significant differences ($P \leq 0.05$) between TRV2::*GFP*::*An2* and TRV2::*CS*::*An2*.

Discussion

VIGS is a high-throughput method for the study of gene function in plants (Baulcombe, 1999). Although VIGS has many advantages as a functional genomics tool, one of the main limitations of VIGS is its irregular distribution of gene silencing in target tissues. To overcome the uneven silencing in target tissues, several studies had developed reporter genes to visualize silenced tissues using transgenic plants of *DEL-ROS* (Becker and Lange, 2010; Fantini et al., 2013; Orzaez et al., 2009) or *GFP* (Quadrana et al., 2011). Using stably expressed transgenes as visible reporter, co-silencing with the reporter and GOI enables us to dissect the precise region where silencing is occurring. The transgenic tomato line expressing two transcription factors *DEL-ROS* driven by ripening-fruit specific promoter ectopically expressed *DEL* and *ROS* in fruit. Furthermore, anthocyanin was accumulated in fruit by up-regulation of the anthocyanin biosynthetic genes. Co-silencing of the reporter gene with GOI in the transgenic plants facilitates observation their phenotype and helps to visualize the silenced region. In *GFP*-silenced regions of *GFP*-overexpressing plant, the fluorescence of *GFP* significantly declined under UV (Quadrana et al., 2011). In this way, target tissues can be easily collected and the effect of the silencing can be better verified with higher phenotypic variation compared to non-collective method. However, this system has difficulty applying in other plants, which are recalcitrant to *Agrobacterium*-mediated transformation. In VIGS, *Agrobacterium*-infiltration works successfully in plant to allow virus expression from plasmids contained in *Agrobacterium*. Virus systemically spreads in plant and silencing of

GOI occurs transiently by siRNA. To extend this concept in other plants such as pepper, I established the endogenous reporter system, *An2*, under natural variation of anthocyanin-rich pepper. Although I intended to develop this system for fruit genetics, the reporter gene-silencing in NMH can easily monitor the silenced region of leaves and flowers and it enables us to collect the tissues without further expression analysis of GOI as demonstrated by co-silencing of *PDS* and *An2* (Figure 3). Therefore, it will be a convenient tool to study pepper genes regulating diverse biological phenomena. In Brand et al. (2014), *CaGLK2* mutant lines with abnormal location of stop codon in the coding region showed pale green fruits and incomplete development of chloroplast. The silencing of *CaGLK2* expressed principally during fruit development with this VIGS system resulted in pale green fruits, a phenotype previously reported in *CaGLK2* mutant lines (Brand et al., 2014). This result suggests that this modified VIGS system is useful to make more precise functional study of genes in pepper fruit.

Previous studies of the *CS* knockdown by VIGS showed reduced capsaicinoid contents in fruits in agreement with this study (Figure 4) (Ogawa et al., 2015; Stewart et al., 2005), clearly indicating that *CS* is responsible for capsaicinoid synthesis. *CS*, originally named as *Pun1*, is believed to catalyze the last step of the biosynthesis although there is no direct evidence that *Pun1* has capsaicin synthase activity. Recently, using a protoplast-based assay for *de novo* capsaicin synthesis and antibodies of the anti-*Pun1*, antagonists of endogenous *Pun1* activity, the *Pun1* gene and its gene product were involved in capsaicin synthesis (Ogawa et al., 2015). Taken together these results, *CS* or *Pun1* primarily control the final step of capsaicinoid biosynthesis.

VIGS system using TRV vector efficiently worked in vegetative tissues of pepper (Chung et al., 2004). However, the silencing signal is not well transmitted to the whole plant, particularly reproductive tissues. Silencing of *PDS* was maintained in flowers and fruits of tomato and enhanced by low temperature and low humidity (Fu et al., 2006). In this study, low temperature was critical requirement of VIGS in fruits of pepper although fruits often resulted in abscission before harvest in all plants (data not shown). Further improvement using different environmental conditions and diverse pepper cultivars should be thoroughly examined. In addition, *An2* as a transcription factor, should not greatly affect the developmental and the biochemical regulation underlying the function of GOI under study, for example, when GOI is a transcription factor or other master regulators as described above. In VIGS, 21-24nt sized siRNA basically targets mRNA of GOI in plant and this matching leads to decrease transcripts of GOI. However, a rare chance of off-target silencing should be considered when a phenotype is observed. To figure out the exact function of GOI, other experiments should be conducted.

This study present the utility of *An2* as use of a endogenous reporter gene in anthocyanin-rich peppers both by down-regulating *PDS* in leaves and *CaGLK2* in fruits and efficiency of monitoring VIGS system. Furthermore, knock down of *CS* in fruits clearly showed the feasibility of this system in metabolic genetics in pepper fruits. These results demonstrate the power of this tool for addressing the roles of regulatory genes in capsaicinoid biosynthesis and secondary metabolism. The application of this system to examine candidate regulatory genes will be useful in the confirmation of candidate genes identified in model species, tomato and Arabidopsis.

LITERATURE CITED

Baulcombe, D.C. (1999). Fast forward genetics based on virus-induced gene silencing. Current Opinion in Plant Biology *2*, 109-113.

Becker, A., and Lange, M. (2010). VIGS--genomics goes functional. Trends Plant Science *15*, 1-4.

Borovsky, Y., Oren-Shamir, M., Ovadia, R., De Jong, W., and Paran, I. (2004). The *A* locus that controls anthocyanin accumulation in pepper encodes a MYB transcription factor homologous to *Anthocyanin2* of Petunia. Theoretical and Applied Genetics *109*, 23-29.

Brand, A., Borovsky, Y., Hill, T., Rahman, K.A.A., Bellalou, A., Van Deynze, A., and Paran, I. (2014). *CaGLK2* regulates natural variation of chlorophyll content and fruit color in pepper fruit. Theoretical and Applied Genetics *127*, 2139-2148.

Burch-Smith, T.M., Anderson, J.C., Martin, G.B., and Dinesh-Kumar, S.P. (2004). Applications and advantages of virus-induced gene silencing for gene function studies in plants. The Plant Journal *39*, 734-746.

Chen, H., Nelson, R., and Sherwood, J. (1994). Enhanced recovery of transformants of *Agrobacterium tumefaciens* after freeze-thaw transformation and drug selection. Biotechniques *16*, 664-668, 670.

Chung, E., Seong, E., Kim, Y.C., Chung, E.J., Oh, S.K., Lee, S., Park, J.M., Joung, Y.H., and Choi, D. (2004). A Method of High Frequency Virus induced Gene Silencing in Chili Pepper (*Capsicum annuum* L. cv. Bukang).

Molecular Cells *17*, 377-380.

Collins, F.S., Green, E.D., Guttmacher, A.E., and Guyer, M.S. (2003). A vision for the future of genomics research. Nature *422*, 835-847.

del Rosario Abraham-Juárez, M., del Carmen Rocha-Granados, M., López, M.G., Rivera-Bustamante, R.F., and Ochoa-Alejo, N. (2008). Virus-induced silencing of *Comt*, *pAmt* and *Kas* genes results in a reduction of capsaicinoid accumulation in chili pepper fruits. Planta *227*, 681-695.

Dinesh-Kumar, S., Anandalakshmi, R., Marathe, R., Schiff, M., and Liu, Y. (2003). Virus-induced gene silencing. Plant Functional Genomics, 287-293.

Dong, Y., Burch-Smith, T.M., Liu, Y., Mamillapalli, P., and Dinesh-Kumar, S.P. (2007). A ligation-independent cloning tobacco rattle virus vector for high-throughput virus-induced gene silencing identifies roles for *NbMADS4-1* and-*2* in floral development. Plant Physiology *145*, 1161-1170.

Fantini, E., Falcone, G., Frusciante, S., Giliberto, L., and Giuliano, G. (2013). Dissection of tomato lycopene biosynthesis through virus-induced gene silencing. Plant Physiology *163*, 986-998.

Fu, D.Q., Zhu, B.Z., Zhu, H.L., Zhang, H.X., Xie, Y.H., Jiang, W.B., Zhao, X.D., and Luo, K.B. (2006). Enhancement of virus-induced gene silencing in tomato by low temperature and low humidity. Molecular Cells *21*, 153-160.

Hammond, S.M., Caudy, A.A., and Hannon, G.J. (2001). Post-transcriptional gene silencing by double-stranded RNA. Nature Reviews Genetics *2*, 110-119.

Han, K., Jeong, H.-J., Sung, J., Keum, Y.S., Cho, M.-C., Kim, J.-H., Kwon, J.-K., Kim, B.-D., and Kang, B.-C. (2013). Biosynthesis of capsinoid is

controlled by the *Pun1* locus in pepper. Molecular Breeding *31*, 537-548.

Kim, S., Park, M., Yeom, S.I., Kim, Y.M., Lee, J.M., Lee, H.A., Seo, E., Choi, J., Cheong, K., Kim, K.T.*, et al.* (2014). Genome sequence of the hot pepper provides insights into the evolution of pungency in *Capsicum* species. Nature Genetics *46*, 270-278.

Kozukue, N., Han, J.-S., Kozukue, E., Lee, S.-J., Kim, J.-A., Lee, K.-R., Levin, C.E., and Friedman, M. (2005). Analysis of eight capsaicinoids in peppers and pepper-containing foods by high-performance liquid chromatography and liquid chromatography− mass spectrometry. Journal of Agricultural and Food Chemistry *53*, 9172-9181.

Liu, Y., Schiff, M., and Dinesh-Kumar, S.P. (2002a). Virus-induced gene silencing in tomato. The Plant Journal *31*, 777-786.

Liu, Y., Schiff, M., Marathe, R., and Dinesh-Kumar, S. (2002b). Tobacco *Rar1*, *EDS*1 and *NPR1/NIM1* like genes are required for N-mediated resistance to tobacco mosaic virus. The Plant Journal *30*, 415-429.

Mazourek, M., Pujar, A., Borovsky, Y., Paran, I., Mueller, L., and Jahn, M.M. (2009). A dynamic interface for capsaicinoid systems biology. Plant Physiology *150*, 1806-1821.

Ogawa, K., Murota, K., Shimura, H., Furuya, M., Togawa, Y., Matsumura, T., and Masuta, C. (2015). Evidence of capsaicin synthase activity of the *Pun1*-encoded protein and its role as a determinant of capsaicinoid accumulation in pepper. BMC Plant Biology *15*, 93.

Orzaez, D., Medina, A., Torre, S., Fernández-Moreno, J.P., Rambla, J.L., Fernández-del-Carmen, A., Butelli, E., Martin, C., and Granell, A. (2009). A visual

reporter system for virus-induced gene silencing in tomato fruit based on anthocyanin accumulation. Plant Physiology *150*, 1122-1134.

Quadrana, L., Rodriguez, M.C., López, M., Bermudez, L., Nunes-Nesi, A., Fernie, A.R., Descalzo, A., Asis, R., Rossi, M., and Asurmendi, S. (2011). Coupling virus-induced gene silencing to exogenous green fluorescence protein expression provides a highly efficient system for functional genomics in Arabidopsis and across all stages of tomato fruit development. Plant Physiology *156*, 1278-1291.

Sarowar, S., Oh, H.W., Cho, H.S., Baek, K.H., Seong, E.S., Joung, Y.H., Choi, G.J., Lee, S., and Choi, D. (2007). *Capsicum annuum CCR4*-associated factor *CaCAF1* is necessary for plant development and defence response. The Plant Journal *51*, 792-802.

Stewart, C., Kang, B.C., Liu, K., Mazourek, M., Moore, S.L., Yoo, E.Y., Kim, B.D., Paran, I., and Jahn, M.M. (2005). The *Pun1* gene for pungency in pepper encodes a putative acyltransferase. The Plant Journal *42*, 675-688.

Tian, S.-L., Li, L., Chai, W.-G., Shah, S.N.M., and Gong, Z.-H. (2014). Effects of silencing key genes in the capsanthin biosynthetic pathway on fruit color of detached pepper fruits. BMC Plant Biology *14*, 314.

Wahyuni, Y., Ballester, A.R., Sudarmonowati, E., Bino, R.J., and Bovy, A.G. (2013). Secondary metabolites of *Capsicum* species and their importance in the human diet. Journal of Nature Products *76*, 783-793.

Wang, M., Wang, G., Ji, J., and Wang, J. (2009). The effect of *PDS* gene silencing on chloroplast pigment composition, thylakoid membrane structure and photosynthesis efficiency in tobacco plants. Plant Science *177*, 222-226.

초 록

Virus-induced gene silencing (VIGS)는 고추와 같이 *Agrobacterium*을 통해 형질전환이 어려운 식물에서 가장 널리 사용되는 중요한 기술이다. VIGS는 유전자 기능 연구의 강력한 기술로써 다양한 식물에서 사용되고 있지만, 바이러스 기반으로 하기 때문에 표현형이 고르지 않는 문제점이 있다. 이 문제점을 해결하고 특히 과실 조직에서 발현되는 유전자의 기능을 연구하기 위해서, 다음과 같은 실험 설계를 하였다. 식물 전체 조직에 안토시아닌이 축적되어 보라색 표현형을 보이는 NuMex Halloween (NMH)을 이용하여 *Tobacco rattle virus* (TRV) 벡터에 안토시아닌 합성을 조절하는 MYB 전사 인자인 *An2*를 삽입하여 시각적 관찰을 용이하게 하였다. NMH에서 TRV-LIC 벡터를 이용하여 *An2*의 발현량을 감소시켰을 때, NMH의 잎, 꽃, 그리고 과일에서 보라색이 사라졌다. TRV-LIC 벡터에 *An2*와 *Phytoene desaturase* (*PDS*)를 나란히 삽입하여 NMH에 감염시킨 결과, *An2*의 발현량 감소로 인한 보라색이 없는 잎과 *PDS*의 발현량 감소로 전형적으로 하얀 잎을 보이는 photobleaching이 나타났다. 반면에 *PDS*만 포함된 TRV 벡터를 감염시킨 식물체의 경우 보라색 잎에 photobleaching 현상만을 보였다. *An2*와 고추 *Golden-like2* (*CaGLK2*)의 발현량을 동시에 감소시킨 식물체에서 과실에서만 안토시아닌이 축적되지 않은 곳에 밝은 초록색을 띠었다. 시각적 관찰을 위한 *An2*와 캡사이신을 최종적으로 합성하는 유전자 *Capsaicin synthase*의 발현량을 동시에 감소시킨 고추 과실에서 보라색이 없어진 초록색 고추의 태좌 만을 수집하여 HPLC를 측정한 결과, capsaicin과 dihydrocapsaicin의 함량이 크게 감소하였다. 이러한 결과들은 *An2*의 발현량 감소로 인한 보라색의 부재를 바탕으로

목표 유전자가 동시에 발현량이 감소된 것을 시각적으로 관찰할 수 있게 해준다. 이로써 이 연구에서 쓰인 VIGS system이 과실에서 발현되는 유전자와 대사산물 합성 관련 유전자의 기능 연구에 중요한 도구로 사용될 수 있음을 시사한다.