FUNCTIONAL CHARACT RPHOGENESIS AND SENESCENCE RELATED GENES USING VIRUS INDUCED GENE SILENCING TECHNIQUE IN *PETUNIA HYBRIDA*

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Abstract

In floricultural crops, flower morphology, such as large petals and double flower formation, and flower longevity are important factors that influence their quality. Petunia has been proved to be an excellent model plant for the study of flower development and senescence. However, even in petunia, there are a lot of genes whose function in flower development and senescence have not yet been characterized. Recently, techniques using virus induced gene silencing (VIGS) have been developed as efficient reverse genetics tools to test gene function. In this study, VIGS system that visualizes silencing induced-flower was established in petunia. Using this system, functional characterization of petunia candidate genes involved in flower morphogenesis and senescence was conducted. In parallel, identification and expression analysis of flower development related-genes that had not yet been identified in petunia was performed.

Disadvantage of VIGS is that silencing is induced in a chimeric manner and it is sometimes difficult to identify flowers on which silencing is induced. To overcome the disadvantage of VIGS, system that use silencing of the chalcone synthase (CHS) gene, one of genes regulating anthocyanin biosynthesis, as a reporter to visualize silencing induced-flower was established. Tandem pTRV2 constructs containing a *Ph-CHS* fragment and target gene fragment(s) are prepared and agrobacterium cultures possessing these constructs are infiltrated onto petunia leaves of 2 to 3 weeks old seedlings. Five different petunia cultivars with blue or purple flowers, 'Cutie Blue', 'Fantasy Blue', 'Picobella Blue', 'Mambo Blue' and 'Mambo Purple' were infected with the construct and differences in silencing patterns was observed among cultivars. In 'Cutie Blue' and 'Fantasy Blue' complete white flowers

were observed as a results of silencing of *Ph-CHS*; however, no white color was noted in flowers or tissues in 'Picobella Blue', 'Mambo Blue' and 'Mambo Purple'. This indicated that effectiveness of this VIGS system differs dependent on flower genetic background. It is also revealed to be dependent on environmental factors such as temperature to get complete silencing phenotypes.

Second, using the VIGS system, redundant function of two C-class MADS-box genes, *pMADS3* and *FBP6* genes on flower morphogenesis was revealed. In flowers induced by either pMADS3-VIGS or FBP6-VIGS, only small changes in commercial appearance were recognized regardless of cultivar, whereas in those induced by pMADS3/FBP6-VIGS, complete conversion of stamens into petaloid tissues and marked enlargement of upper limb-like tissues were observed, resulting in a decorative appearance in all the four cultivars. Moreover, cultivar-dependent conversion of carpels into new flowers was noted in pMADS3/FBP6-VIGS flowers. Of the four cultivars, only 'Mambo Purple' exhibited the development of new flowers instead of carpels and the emergence of ectopic new flowers from the axil of petaloid organs, which created an ornamental appearance with a high commercial value. Further, investigation of large and small petaloid stamens induced by pMADS3/FBP6-VIGS and pMADS3-VIGS, respectively, revealed only small differences in cell size compared to the large difference in total surface area indicating that the size of petaloid stamens in C-class genes suppressed flower was determined at early stage of flower development and the suppressed C-class gene function at the late stage of flower development has little influence on the final size of petaloid tissue.

Third, function of genes encoding ethylene signal components, Ethylene Insensitive 2 (EIN2) and EIN3 like (EIL), on flower senescence was characterized. A cDNA encoding EIN2 (*Ph-EIN2*) and five cDNAs encoding EILs (*Ph-EIL1* to *5*) were cloned from petunia petals and VIGS of these genes together with *Ph-CHS* were conducted. VIGS of all *Ph-EILs* were attempted at once by using a conserved region of the *Ph-EILs*. The flower longevity of VIGS-induced flowers was compared to those of non-VIGS flowers under the condition with or without pollination. The results showed prolonged longevity of VIGS-induced flowers indicating the involvement of *Ph-EIN2* and *Ph-EILs* on petal senescence. When flowers were treated with propylene, an ethylene analogue, marked delay in petal senescence was observed in Ph-EIN2-VIGS flowers in comparison to non-VIGS flower. Thus, it has been clarified that *Ph-EIN2* is functioning as ethylene signaling factor and involved in the petal senescence.

Fourth, petunia orthologues of pollen formation-related genes *Tapetum Determinant 1* (*Ph-TPD1*) and *Excess Male Sporocytes 1* (*Ph-EMS1*) were identified and their expression patterns during flower bud development were determined. Quantitative real time analysis of *Ph-TPD1* in stamen of flower buds at four different developing stages (5 mm to 15 mm in length buds) showed that *Ph-TPD1* expression can be detected only in the youngest stage. Expression of Ph-EMS1 was detected in stamen at all stages with highest expression at the youngest stage. Analysis in various tissues of the buds (sepal, petal, stamen and style) at youngest stage showed that *Ph-TPD1* and *Ph-EMS1* are expressed not only in stamen but also in developing pistils and petals. In sepal, expression of *Ph-TPD1* was not detected, whereas expression of *Ph-EMS1* was detected at lower level than in the other tissues. These results

suggest that *Ph-TPD1* and *Ph-EMS1* cloned in this study interact with each other to regulate early steps of pollen development. It is also indicated that they are involved in development of style.

The findings obtained in this study would be valuable for breeding new cultivars and developing technology to improve quality of petunia and other floricultural crops.

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General Introduction

Petunia belongs to the Solanaceae, which is a plant family of great economic importance. Solanaceaous species are used for food (e.g., potato, tomato, pepper, eggplant), as drugs (e.g., tobacco, deadly nightshade, mandrake), and as ornamentals (e.g., petunia, velvet tongue, Datura spp., Schizanthus spp.) (Knapp et al.,2004). In 1803, Jussieu established the genus Petunia (Solanaceae), later referred to as Petunia sensu Jussieu. Petunia is an excellent landscape plant because of its great ornamental values apart from rose, carnation, begonia, lily, chrysanthemum and others. Ornamental plants are plants that are grown for decorative purposes in gardens and landscape design projects, as houseplants, for cut flowers and specimen display. So it is important to study on mechanism(s) leading to improving quality of this flower. While there are comparables, quality can be perceived from many aspects like flower shapes, sizes, colors, longevity and others. Flower longevity is one of important factors that determined quality of ornamental plants. The senescence of many flowers, including petunia is controlled by the gaseous plant hormone ethylene. Senescence of petunia flowers is accelerated by the gaseous plant hormone ethylene. Senescence of petunia flowers is accelerated by exogenous ethylene treatment or pollination through an induction of autocatalytic athylene biosynthesis (Gilissen and Hoekstra, 1984; Tang and Woodson, 1996). Research had been reveal that flower senescence were accelerate by pollination and fertilization. Treating petunia flower with ethylene accelerates corolla senescence and expression of senescence related genes. In the case of the longest lived flowers it is perhaps not surprising that flower longevity may be modified by environmental factors or more dramatically by fertilization or pollination. To achieve and obtain most of the quality traits we desire to study about flower morphology, ethylene signal pathway and pollen formation related genes. Petal senescence in petunia is accompanied by decreased nucleic

acid and protein content, DNA and nuclear fragmentation, and structural and compositional changes in the plasma membrane. Pollen development in angiosperms is one of the most important processes controlling plant reproduction. When no pollen plants are bred, it will improve a lot in our daily life. For example, double flower plant without pollen would be expected to have long live vase flower, much easier to be passed safety test for transgenic flower plant, helps florist from removal pollen flower in cased lily, less pollen allergy fever and our clothes will not dirty with pollen stain. From all these benefits it will be important to study about flower development and pollen development in petunia.

Flower morphology

The diversity of angiosperm flowers on earth is tremendous, there are over 300,000 different species, each with a unique flower. Yet, all flowers serve a similar function and evolved from the same common ancestor. Therefore, it has been considered by understanding a few basic structures in a "general" flower, we can understand the structure and function of most flowers. In petunia there are typical flower morphology consists of four organs: sepals, petals, stamens and pistils (including carpels and ovules). What organ is formed and where each organ is specified are determined by a combinatorial action of five functional classes of genes. This was first formulated in the famous ABC model, which has formed the foundation for our understanding of floral development, and was later extended with two extra functional classes D and E (Angenent et al., 1994, 1995; Coen and Meyerowitz, 1991; Honma and Goto, 2001; Pelaz et al., 2001). Perfect flowers contain four types of floral organ arranged in four concentric rings, known as whorls. The four organ types are sepals (outermost or whorl 1), petals (whorl 2), male reproductive stamens (whorl 3) and female reproductive carpels (innermost or whorl 4). The ABC model proposed that three functions, A, B and C, each defined by a class of homeotic mutant found in both *Arabidopsis* and *Antirrhinum*, specify

the organs that form in the four whorls of the flower. Expression of C-class gene alone in whorl four causes carpels formation. In whorl 3 both B- and C-class genes are expressed, which specifies stamens. Petals are formed in whorl 2 due to concomitant expression of A- and B-functions and expression of A-class gene alone in whorl 1 results in sepals formation. Almost all of the identified players in this model belong to closely related paralogous lineages of the MADS-box gene family. There are a lot of genes in petunia whose function in flower morphology have not yet been characterized.

Ornamental floral morphologies, such as double flowers and large flower size, have been selected preferentially during the breeding of wild plants to produce floricultural plants. It is also a way to sustaining flower longevity because flower pollination will not occur and ethylene will not induced to accelerate flower senescence. As reviewed by Nishijima (2012), four different morphological changes induce double flowers: (1) conversion of the stamens and carpels into petals; (2) simultaneous increase of petals and other floral organs including stamens, sepals, and carpels; (3) increase of ray florets in the capitulum; and (4) paracorolla development. However, only conversion of the stamen and carpel into petals is popularly studied and regularly used. Conversion of the stamens and carpels into petals is the most common morphological change and they were observed in various floricultural plants, such as rose, stock, peony, and Japanese morning glory (Saito, 1959). The double flower form in petunia is created through the formation of multiple layers of corolla (i.e. petal structures) and s associated with floral organ changes. *AGAMOUS (AG)* is a class-C floral homeotic gene hat is involved in specification of stamens and carpels formation. In petunia, two genes share igh sequence similarity with the *Arabidopsis AG* gene, *pMADS3* and *FBP6*. The *pMADS3* o-suppression has been reported to result in conversion of stamen into petaloid structures Kapoor et al., 2002). However, the homeotic conversion was not so remarkable as shown in

Arabidopsis, which lead us to assume that *FBP6* may act redundantly with *pMADS3* on the determination of stamens and carpels.

Ethylene signaling pathway.

Ethylene is a plant hormone involved in the regulation of a large number of processes from seed germination, root hair development, root nodulation, flower senescence, abscission, and fruit ripening (Johnson and Ecker, 1998).Significant progress toward the delineation of ethylene action in plants has been made during the past decade using a combination of genetic and molecular-biology approaches (Guo and Ecker, 2004). This work has made the ethylene pathway one of the most well-defined signaling pathways in plants. Great progress in understanding the mechanisms by which plants respond to ethylene was achieved through using *Arabidopsis thaliana* as a model plant.

Since the first ethylene receptor *ETR1* was cloned from *Arabidopsis* (Chang et al. 1993), a family of genes that encode four other ethylene receptors (*ETR2, EIN4, ERS1,* and *ERS2*) was identified (Hua et al. 1995, 1998; Sakai et al. 1998). Knockout of multiple ethylene receptors conferred stronger constitutive ethylene responses (Hua and Meyerowitz 1998), indicating that the ethylene receptors are negative regulators and the family members function redundantly. Ethylene receptors function as negative regulators through constitutive triple response 1 (*CTR1*), another genetically identified negative regulator, which was identified from a screen for constitutive ethylene-response mutants resulting from loss-of-function ctr1 alleles (Kieber et al. 1993). *CTR1* is a Raf-like (MAPKKK) kinase that has serine/threonine kinase activity (Huang et al. 2003). Physical interaction of the *CTR1* with ETR1 was demonstrated by both in vitro and in vivo assays (Clark et al. 1998; Gao et al. 2003). This interaction mostly occurs at the endoplasmic reticulum (ER) membrane (Chen et

al. 2002; Gao et al. 2003). Downstream of *CTR1* in the ethylene signaling pathway is *EIN2*, which was initially isolated through genetic screens for ethylene-insensitive mutants (Guzmán and Ecker 1990). Overexpression of the C-terminus of *EIN2* leads to constitutive ethylene responses (Alonso et al. 1999). EIN2-interacting proteins (ETP1 and ETP1) contain an F-box domain, which facilitates the ubiquitylation of *EIN2* and regulates its degradation by the 26S proteasome pathway (Qiao et al. 2009). *EIN2* directly regulates *EIN3*, which functions as a transcription factor that binds to the primary ethylene response element (PERE) of *ERF1* and consequently induces tne expression of the downstream ethylene-response genes (Solano et al. 1998). *EIN3* is a member of a family of proteins, which includes EIN3-like (EIL) proteins (Chao et al. 1997). EIN3 protein levels are regulated by two F box proteins, EBF1 and EBF2, in a ubiquitin/proteasome pathway (Guo and Ecker 2003; Potuschak et al. 2003; Gagne et al. 2004; Binder et al. 2007; An et al. 2012b). EIN3 can directly target the promoter of ERFs and the expression of the downstream genes is responsive to various stresses (Zhang et al. 2009, 2011; Zhu et al. 2010; Xu et al. 2011).

In petunia the role of *Ph-EIN2* was analyzed in a wide range of plant responses to ethylene (Shibuya et al. 2004) *Ph-EIN2* mRNA was present at varying levels in tissues examined, and the *Ph-EIN2* expression decreased after ethylene treatment in petals. These results indicate that expression of *Ph-EIN2* mRNA is spatially and temporally regulated in petunia during plant development. Transgenic petunia plants with reduced *Ph-EIN2* expression were compared to wild-type and ethylene-insensitive petunia plants expressing the *Arabidopsis* etr1-1 gene for several physiological processes (Shibuya et al. 2004). Both *Ph-EIN2* and *etr1-1* transgenic plants exhibited significant delays in flower senescence and fruit ripening, inhibited adventitious root and seedling root hair formation, premature death, and increased hypocotyl length in seedling ethylene response assays compared to wild-type.

Moderate or strong levels of reduction in ethylene sensitivity were achieved with expression of both *etr1-1* and *Ph-EIN2* transgenes, as measured by downstream expression of *Ph-EIL1*. These results demonstrate that *Ph-EIN2* mediates ethylene signals in a wide range of physiological processes and also indicate the central role of *EIN2* in ethylene signal transduction.

Ethylene Insensitive 3 (*EIN3*) and EIN3 like (*EIL*) are transcription factors functioning redundantly for transduction of ethylene signal. However, characterizations of gene families that act in a redundant manner have not yet been progressed on research of flower senescence in petunia. So that it will be interesting to analysis functions of *Ph-EIL* family on flower senescence.

Pollen formation-related genes

Pollen development in angiosperms is one of the most important processes controlling plant reproduction. At the same time, controlling pollen development also helps increasing commercial values by sustaining flower quality and longevity. It is also useful for avoiding pollen stains. In flowering plants, anthers, the male parts of flowers, contain reproductive cells that generate pollen (pollen mother cells) and somatic tissues that are required for pollen development and release (tapetum, middle layer and endothecium). The anther contains both reproductive and non-reproductive (somatic) cells and its development has been divided into 14 stages according to morphological features (Sanders et al., 1999). At stage 1, the anther contains three cell layers, L1, L2 and L3. The L1 and L2 layers form the epidermis and archesporial cells, respectively, and the L3 layer gives rise to the vascular and connective tissues. At stage 2, the archesporial cells further divide into primary parietal and primary sporogenous cells to form the stage 3 anther. The primary parietal cell layer divides again to form two layers of secondary parietal cells. At stage 4, further division and differentiation of

secondary parietal cells generate the endothecium, middle layer and tapetum. The primary sporogenous tissue gives rise to microsporocytes at stage 5, and these undergo meiosis to form microspores during stages 6–8. With progress of studies on pollen formation in *Arabidopsis*, several genes that are involved in regulation of pollen development have been identified. Those include genes encoding *AG (Agamous)* MADS-box transcript factor, *TPD1* (*Tapetum Determinant 1*) and *EMS1* (*Excess Microsporocytes 1*).

Virus Induced Genes Silencing (VIGS) techniques

Recently, techniques using virus induced gene silencing (VIGS) have been developed as efficient reverse genetics tools to test gene function. VIGS technologies have multiple advantages. They are easy, convenient and fast to be implemented; thus, they are especially useful for analysis of gene families that act in a redundant manner and require a large number of silencing transformants. VIGS can induce tissue and stage specific silencing even without using specific promoter, which makes it possible to observe phenotype (gene function) that cannot be seen in knock-out plant and stable RNAi plant with constitutive promoters.

On the other hands, the disadvantage of VIGS is that silencing is induced in a chimeric manner. Therefore, it is sometimes difficult to characterize function of a particular gene, when it is involved in phenotype that is not apparently observed. In case no expected phenotypic change was observed, we cannot even figure out whether that is due to unsuccessful silencing resulted from technical problems or the target gene really does not have expected function.

In this study, I would like to introduce a VIGS system to overcome these disadvantages. Then, using this system, I attempted to studies on genes related to flower morphogenesis and flower senescence in petunia.

Aim of this study

In this study, first I performed VIGS technique using silencing of chalcone synthase (CHS) gene as a reporter to focus on characterization of gene families that act in redundant manner that related to flower quality in petunia.

Second, the relation between petunia A-class gene and C-class genes on performing double flower. Furthermore, I cloned and analyzed the expression patterns of A and C-class genes on conversion stamen into petaloid stamen in petunia. Based on these results, the role of these genes in regulating development of the stamen into a petaloid organ is discussed.

Third, functional analysis of genes related to petal senescence and flower longevity, Ethylene Insensitive 2 (EIN2) and EIN3 like (EIL) genes in petunia.

Fourth, focusing on pollen formation-related genes TAPETUM DETERMINANT1 (TPD1) and EXCESS MALE SPOROCYTES1 (EMS1) were cloned and their expressions during pollen development were studied.

Chapter 1

Virus Induced Genes Silencing (VIGS) System Using CHS as a reporter in different *Petunia Hybrida* cultivars

1.1 Introduction

Gene silencing is the process of "turning off" a gene, thereby preventing it from expressing in the form of protein production or other forms of expression. This process happens naturally in many cases for the purpose of regulating the expression of genes and preventing potential damage from viruses. Gene silencing is also an important laboratory technique, as disabling a gene is a highly effective way for characterising function of that gene. Silencing a gene and observing the changes in phenotypes is one valuable way to determine the exact role of a given gene.

Forward genetics is traditional approach to study gene functions. Reverse genetics is based on the genome sequence, bioinformatics and molecular techniques to study the function of genes. Stable transformation is a frequently used method to overexpress and suppress target genes in plant; however, time and labor consuming are the drawbacks of this approach. These problems could be solved by a transient method. Knock-down or knock-out of gene expression is a crucial method for studying gene function. At least four methods have been developed and applied in this field: hairpin RNA interference (hpRNAi), artificial micro RNAs (amiRNA), miRNA-induced gene silencing (MIGS) and virus-induced gene silencing (VIGS) (de Felippes et al. 2012).Each method has its own advantages as well as disadvantages. VIGS has been seen as a promising reverse genetics tool, especially for non-

model plant species (Burch-Smith et al. 2004). Successful application has been demonstrated in more than 30 plant species (Becker and Lange 2010).

VIGS, a transient method, uses virus as a vector to trigger a natural defense mechanism, post-transcriptional gene silencing (PTGS), in plants (Voinnet 2001, Lu et al. 2003).Double-stranded RNA, generated by activity of virus or host RNA-dependent RNA polymerase (RDR/RdRp) in RNA viruses (Soosaar et al. 2005), or complementary RNA strands in DNA viruses (Voinnet 2005), is recognized by Dicer-like proteins (DCL). DCL ribonuclease cleaves viral doubled-stranded RNA into small fragments, 21–24 base pairs in length, which are called short-interfering RNAs (siRNAs). Consequently, single strand RNA, which is protected by the methyl group on the 3'-terminal nucleotide added by the methyltransferase HUA ENHANCER 1 (HEN 1) (Brodersen and Voinnet 2006), is incorporated into an Argonaute (AGO) protein to form RNA-induced silencing complex (RISC) (Ding and Voinnet 2007). This complex will cleave RNA homologous with the guide strand siRNA and lead to PTGS. Moreover, the effect of RNA-silencing reactions is amplified and spread systemically in plant by recruiting host RDR6 and other components (Brodersen and Voinnet 2006). Kumagai et al. (1995) developed, for the first time, *Tobacco mosaic virus* (TMV) as a vector to knock down the endogenous *PDS* gene in *Nicotiana benthamiana* based on this mechanism. From that, VIGS has been developed by incorporating the viral genome as cDNA into the T-DNA region of *Agrobacteium tumerfaciens* Ti-plasmids for transient transformation. Within the viral genome, plant endogenous target genes will be knocked down by PTGS (Stratmann and Hind 2011). More than 34 RNA and DNA viral genomes (Senthil-Kumar and Mysore 2011) have been developed to generate VIGS systems, in which *Tobacco rattle virus* (TRV) is most widely used and successfully applied to several plant species.

Two positive-sense, single-stranded RNA genomes, a broad range of hosts, systemic movement, and mild symptoms (Ratcliff et al. 2001, Macfarlane 2010) are the advantages, which TRV has been chosen to develop VIGS vector system at the beginning of the 2000s (Ratcliff et al. 2001, Liu et al. 2002). In these systems, cDNA of TRV RNA1 and RNA2 are separately cloned in different *Agrobacterium* binary plasmids under the 35S promoter to produce pTRV1 and pTRV2, respectively. They then will be transformed into bacteria and infiltrated into plants through the mixing of two bacterial cultures. The non-structural genes in TRV RNA2 construct were replaced by a multiple cloning site where the host target gene will be inserted to trigger PTGS (Ratcliff et al. 2001, Liu et al. 2002). TRV based VIGS vector, which developed by Liu et al. (2002), has been successful applied in model and crop plants such as tomato, *Nicotiana benthamiana* and several Solanaceous species (Burch-Smith et al. 2004, Senthil-Kumar et al. 2007), poppy (Hileman et al. 2005, Wege et al. 2007), *Arabidopsis* (Burch-Smith et al. 2006), *Aquilegia* (Gould & Kramer 2007), *Thalictrum dioicum* (Di Stilio et al. 2010), *Jatropha curcas* (Ye et al. 2009), cotton (Gao et al. 2011), strawberry (Jia et al. 2011), *Cysticapnos vesicaria* (Hidalgo et al. 2012). It has been shown to be a powerful tool for studying the flower development in emerging model plant systems (Di Stilio 2011) and plant metabolic pathways (Burch-Smith et al. 2004). This system, besides the advantages, there are some limitations such as interference from viral symptoms with interpretation of data, off-target silencing, and low silencing efficiency, which need to be overcome to obtain meaningful results (Senthil-Kumar and Mysore 2011).

VIGS technologies have multiple advantages. They are easy, convenient and fast to be implemented; thus, they are especially useful for analysis of gene families that act in a redundant manner and require a large number of silencing transformants. On the other hands, the disadvantage of VIGS is that silencing is induced in a chimeric manner. Therefore, it is

sometimes difficult to characterize function of a particular gene, when it is involved in phenotype that is not apparently observed. In case no expected phenotypic change was observed, we cannot even figure out whether that is due to unsuccessful silencing resulted from technical problems or the target gene really does not have expected function. Here, I would like to introduce a VIGS system using chalcone synthase (CHS) as a reporter which describe in Chen et al. (2004) to overcome these disadvantages.

In this study, we apply VIGS system with CHS as a reporter to analysis efficiency of silencing in five different cultivars in petunia. We also used protein blotting to detect virus efficiency in an early development of VIGS plants.

1.2 Materials and methods

Plant materials and growth conditions

Petunia (*Petunia hybrida*) seeds of cultivars 'Fantasy Blue' and 'Picobella Blue' were obtained from Sakata Seed Co. (Yokohama, Japan), whereas those of 'Cutie Blue', 'Mambo Blue' and 'Mambo Purple' were obtained from Takii Seed Co. (Kyoto, Japan). Plants were grown in an isolated greenhouse under natural day and night cycles with a day/night temperature regime of 25 °C/20 °C, respectively.

Plasmid construction

We refer method describe in Chen et al. (2004) in which silencing of "chalcone synthase gene" (*Ph-CHS)* were used as a reporter to visualize silencing induced-portions. The tobacco rattle virus (TRV)-based VIGS system that uses the suppression of the anthocyanin pathway via chalcone synthase silencing as the reporter (Chen et al., 2004) was introduced in this study. The pTRV1 and pTRV2 VIGS vectors were kindly provided by Dr. Savithramma Dinesh-Kumar, Yale University (Liu et al., 2002). A cDNA fragment of petunia chalcone synthase, Ph-CHS, was amplified and cloned into the EcoR1 site of pTRV2 vector to form pTRV2 PhCHS as reported by Chen et al. (2004).

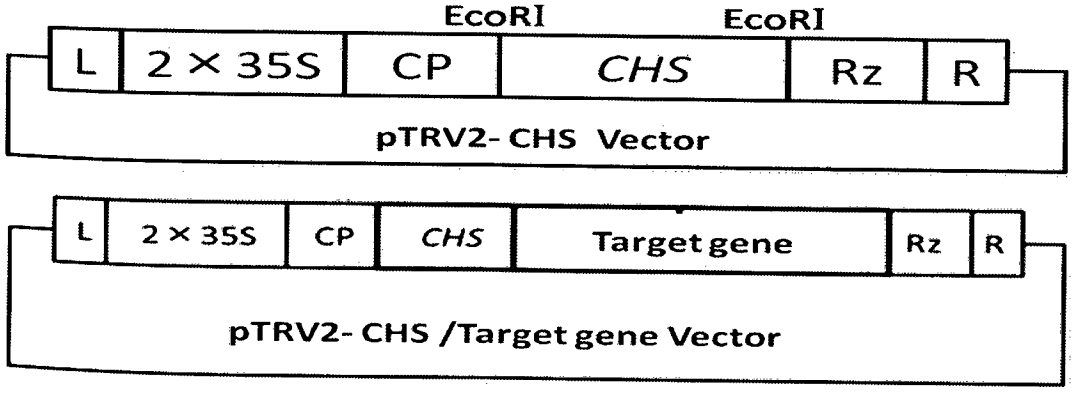

Fig. 1.2 Vector construct of pTRV2-CHS for VIGS (virus induced genes silencing).

The non-conserved regions of petunia C-class genes, pMADS3 and FBP6, were amplified using the primers listed in Table 2.1 and cloned into the SmaI site of pTRV2 PhCHS vector individually to generate constructs for silencing pMADS3 and FBP6 separately. To generate a construct for silencing both pMADS3 and FBP6 simultaneously, pMADS3 and FBP6 fragments were fused and cloned into the SmaI site of pTRV2 PhCHS vector.

Agroinoculation of TRV vectors

Agrobacterium cultures possessing was grown overnight at 28 °C in YEP medium with appropriate antibiotics. Then, the cells were harvested and resuspended in inoculation buffer (10 mM $MgCl_2$, 10 mM MES, 200 μM acetosyringone) to an O.D. of 2.0 and incubated at room temperature for 3 h. The bacteria containing pTRV1 were mixed with the bacteria containing pTRV2 derivative in a 1: 1 ratio. These constructs are infiltrated onto petunia leaves of 2 to 3 weeks old seedlings using a 1 ml disposable syringe without a needle. Suppression of the anthocyanin pathway via *Ph-CHS* silencing allows easy visual identification of silencing flowers and/or flower-sections. Effects of the VIGSs of target genes can be investigated by comparing white (silenced) and purple (nonsilenced) flowers on the same plant.

Protein blotting to detect virus efficiency after inoculation

Sample of infection leaves was collected after 1 month inoculation by VIGS and put into the 1.5ml tube. The 0.1 g of leaves sample were grown in liquid nitrogen and resuspend into 100ul PBS buffer. Membrane were wet in PBS buffer for 5 min and placed onto manifold and clamp pod into place following manufacturer's instructions (Bio-Dot apparatus Bio Rad). RNA sample were applied into well and then the casset well vacuum until RNA samples were filter through membrane completely. The blotted membrane were dried

completely and then washed with methanol and then PBST buffer for 5min each. Membrane were then washed with 2% tryton for 30 min until the solution were cleared. The blots were immersed in the blocking solution (3% PBST milk) for 30 min with occasional shaking at room temperature while paying attention that the reagent solution keep covering the blot with the solution. The membrane were washed twice with PBS-Tween buffer for 10 min each, then, incubated again with anti-Rabbit +AP second antigen diluted in PBS-Tween for 60 min with occasional shaking at 37℃. After washing twice with PBS-Tween buffer for 10 min each, the blot were incubated with buffer 3 (0.1M Tris-HCl, 0.1M NaCl pH9.5) for 5min to make the membrane became alkali. Soak blotted membrane in substrate solution (chromogen) 5–10 min at 37℃ to detect enzyme activity. Rinse blotted membrane for a few seconds in distilled water to stop reaction. After washing and drying, color of stained membranes were visually observed.

1.3 Results and Discussions

Silencing of chalcone synthase (CHS) gene as a reporter

In this study, suppression of the anthocyanin pathway via chalcone synthase silencing was used as the reporter for easy visual identification of silenced flowers/tissues in petunia. Differences in silencing patterns have been observed among petunia cultivars. CHS silencing flower showed three patterns of flower white flower (silence), mix flower (silence) and original flower (non silence) (Fig. 1.3). VIGS treatment using CHS as a marker were conducted in five petunia cultivars, 'Cutie Blue', 'Fantasy Blue', 'Picobella Blue', 'Mambo Blue' and 'Mambo Purple'.

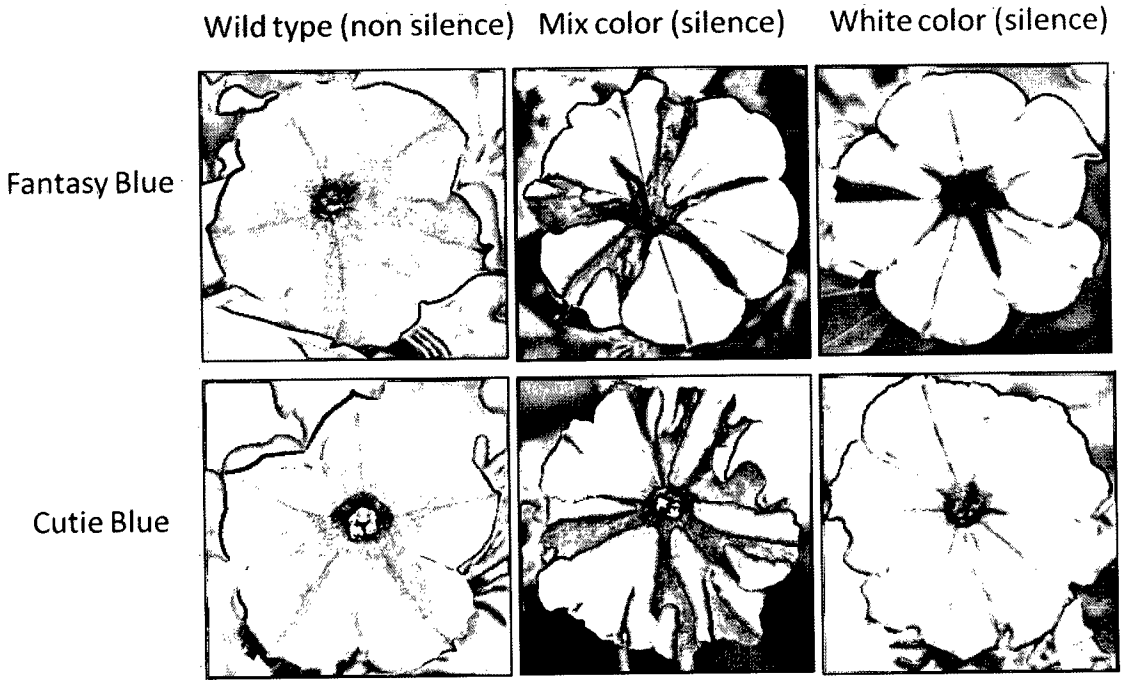

Fig. 1.3 Flower patterns of chalcone synthase (CHS) gene silencing in Fantasy Blue and Cutie Blue cultivar.

In 'Picobella Blue', 'Mambo Blue' and 'Mambo Purple', however, no white color was noted in flowers or tissues, perhaps due to an unknown genetic background as reported by Chen et al. (2004). In 'Cutie Blue' and 'Fantasy Blue', on the other hand, completely white flowers were observed (Fig. 1.3), indicating the strong and complete silencing in those cultivars. This result showed that 'Cutie Blue' and 'Fantasy Blue' are a good CHS silencing flower rather than 'Picobella Blue', 'Mambo Blue' and 'Mambo Purple'. Our research confirmed that 'Cutie Blue' is one of the best cultivar for VIGS treatment using CHS as a marker for silencing. Fantasy Blue and Cutie Blue may be breeded from a similar genetic background and are morphologically indistinguishable. The identification of genetic differences in these gene families could be used inbreeding programs to create cultivars that would be ideal for VIGS-based studies.

Cultivar dependent differences in VIGS Silencing of CHS and target genes

Five cultivars were evaluated to determine differences in silencing when using *CHS* as a marker with target gene. We conducted *CHS* silencing with C-class genes (*pMADS3* and *FBP6*) as a target genes. Our results indicate that all cultivars performing double flower but, only Fantasy Blue and Cutie Blue showed complete white double flower. For Picobella Blue, Mambo Blue and Mambo Purple complete double flower with original flower color were observed **(Fig. 1.4)**. We even observed no white spot of *CHS* silencing and no flower with mix color in this three petunia cultivars. At this point the reason cannot be proved. There must be another factor that controlling *CHS* silencing and/or antocyanin accumulator.