FUNCTIONAL CHARACTERIZATION OF FLOWER MORPHOGENESIS AND SENESCENCE RELATED GENES USING VIRUS INDUCED GENE SILENCING TECHNIQUE IN *PETUNIA HYBRIDA*

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Functional characterization of flower morphogenesis and senescence related genes using virus induced gene silencing technique in *Petunia Hybrida*

A dissertation submitted by Siti Hajar Noor Binti Shaarani in partial fulfilment of the requirements for the Doctor of Philosophy in Agriculture in the Graduate School of Natural Science and Technology, Okayama University, Japan.

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Declaration

This thesis does not incorporate without acknowledgement any material previously submitted for a degree, diploma or fellowship or any other titles in any university, college or other learning institutions. To the best of my knowledge and belief, it is a true record of original research work done by the candidate Siti Hajar Noor Binti Shaarani at the Okayama Graduate School of Natural Science and Technology, and accepted for the award of the Doctor of Philosophy in Agriculture of the Okayama University, Japan.

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In the name of ALLAH, The Most Gracious and Most Merciful

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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, \textit{pMADS3} and \textit{FBP6} genes on flower morphogenesis was revealed. In flowers induced by either \textit{pMADS3-VIGS} or \textit{FBP6-VIGS}, only small changes in commercial appearance were recognized regardless of cultivar, whereas in those induced by \textit{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 \textit{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 \textit{pMADS3/FBP6-VIGS} and \textit{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 \textit{Ph-TPDI} and \textit{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. Solanaceous 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 ethylene 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
is associated with floral organ changes. AGAMOUS (AG) is a class-C floral homeotic gene
that is involved in specification of stamens and carpels formation. In petunia, two genes share
high sequence similarity with the Arabidopsis AG gene, pMADS3 and FBP6. The pMADS3
co-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 the 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 \textit{etr1-1} and \textit{Ph-EIN2} transgenes, as measured by downstream expression of \textit{Ph-EIL1}. These results demonstrate that \textit{Ph-EIN2} mediates ethylene signals in a wide range of physiological processes and also indicate the central role of \textit{EIN2} in ethylene signal transduction.

Ethylene Insensitive 3 (\textit{EIN3}) and EIN3 like (\textit{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 \textit{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 tumefaciens* 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).

![Diagram](image)

**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 100µl 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°C. 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°C 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’.

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**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 bred from a similar genetic background and are morphologically indistinguishable. The identification of genetic differences in these gene families could be used in breeding 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.
VIGS efficiency depends on viral proliferation and systemic movement through-out the plant. Although tobacco rattle virus (TRV)-based VIGS has been successfully used in petunia (*Petunia × hybrida*), the protocol has not been thoroughly optimized for efficient and uniform gene down-regulation in this species. The ability and extent of the virus to elicit PTGS can vary greatly and differs with environmental conditions and inoculation methods (Lu et al.2003). The location and amount of gene silencing within a plant can be highly variable when using viruses to induce silencing (Chen et al.2004; Jiang et al.2008).

**Fig. 1.4** Flower patterns of chalcone synthase (CHS) gene silencing with C-class genes (pMADS3 and FBP6) in Picobella Blue, Mambo Blue and Mambo Violet cultivar.
**Long term VIGS silencing in petunia**

To determine whether CHS silencing persist for a long time, we observed CHS silencing with pMADS3 and FBP6 genes for a year after inoculation on January 2012 in Cutie Blue cultivar. Our observation showed that, after 3 month inoculation plant were bloomed with white, mix and purple double flowers (Fig. 1.5). The double flowers were bloomed until early summer but after that double flower stop blooming. However, six month after inoculation double flower started blooming again on early winter with more white and mix double flower compared to before (Fig. 1.5). Our finding revealed that VIGS silencing can persist for a long term in petunia which was consistent with the recent finding of Senthil-Kumar and Mysore et al (2011) on PDS silencing in *Nicotiana benthamiana* and tomato. In addition, this finding suggest that CHS silencing are more efficient in low temperature, consistent with the finding of Shaun et al (2013) in which 20°C day/18 °C night temperatures induced stronger gene silencing than 23 °C/18 °C or 26 °C/ 18 °C. It is conclude that temperature and environmental factors can affect gene silencing efficiency.

![Fig. 1.5 Morphologically changes of CHS silencing flower related to temperature in Cutie Blue cultivar](image-url)
Western dot blot analysis to determine VIGS efficiency after inoculation

After 1 month inoculation leaves sample were collected and crude protein extract was blotted on the membrane for detection of TRV virus with anti-Rabbit +AP. By this process I can detected which plant are successfully infected and which not infected. As shown in Fig. 1.6 spots with positive reaction (clear purple color) indicate infected plants while spots with negative reaction (no purple color) indicate non-infected plants. Based on this result, infected plants were selected for further growth. This can helps us from wasting our time by waiting not silenced plant and also decrease places for growing many plants. Thus, it will help us for growing a lot of VIGS plants in a short time.

Fig. 1.6 Detection of pTRV1 virus in petunia silencing leaf samples using pTRV1 anti-rabbit by protein blotting.
1.4 Summary

This chapter investigated VIGS system using CHS as a reporter to detect silencing parts via suppression of the anthocyanin pathway. The following points emerged from the present results: 1) Silencing of *chalcone synthase* (*CHS*) gene as a reporter can be used to detect silencing parts in petunia. 2) Differences in silencing patterns have been observed among five petunia cultivars. ‘Cutie Blue’ was best material for this system. 3) Based on the results in C-class gene silencing efficiency of *CHS* silencing depends on flower genetic background, temperature and also environmental factors. It was also revealed that VIGS silencing persist in the long term in petunia. 4) Western dot blot analysis to detect TRV viruses can be useful for determining efficiency of VIGS in an early stage developmental of silencing plant. It is valuable method because it is simple, economical, rapid and sensitive. The technique also appears to be a useful and practical diagnostic technique to detect virus infected plant.
Chapter 2

Double Flower Formation Induced by Silencing of C-class MADS-Box Genes and Its Variation among Petunia Cultivars

2.1 Introduction

The ability to bear double flowers is an important trait that enhances the commercial value of floricultural plants, including *Petunia hybrida*, because it produces a voluminous appearance. Breeding with double flower mutants is generally carried out to obtain double flower cultivars. However, mutant phenotypes are usually unstable and long periods are required to fix stable phenotypes (Akita et al., 2011; Nishijima, 2012). In particular, flowers having large petaloid tissues and a decorative voluminous appearance are difficult to breed. Thus, elucidation of the mechanism(s) underlying the generation of double flowers with a decorative voluminous appearance is required to develop efficient breeding methods that employ molecular tools.

One of the morphological changes that result in double flower formation is the conversion of stamen and carpel into petal and new inflorescence, respectively. Those changes are induced by suppressing the functions of C-class floral homeotic genes. The framework for floral organ identity is well illustrated by the classic ABC model, where combinational expression patterns of three classes of floral homeotic genes specify the development of floral organs (Bowman et al., 1991; Coen and Meyerowitz, 1991; Rijpkema et al., 2007). The combined expression of A- and B-class genes in whorl 2 leads to a petal, the expression of B- and C-class genes in whorl 3 leads to a stamen, and the expression of C-class genes alone in whorl 4 leads to a carpel. A- and C-class genes repress each other’s
expression to avoid the overlap of A- and C-class gene expression (Drews et al., 1991; Gustafson-Brown et al., 1994). Therefore, it is considered in the classic ABC model that suppressing C-class genes in whorl 3 would result in the expression of A-class genes in this area, which in turn would lead to the conversion of stamen into petal, although recently, researchers have started to question the function of A-class genes (Causier et al., 2010; Heijmans et al., 2012b). Furthermore, as C-class genes are required for the control of floral meristem determinacy, the suppression of C-class genes would induce the indeterminate development of flowers in whorl 4 (Lenhard et al., 2001; Lohmann et al., 2001).

C-class genes belong to the \textit{AG-clade} of the large MADS-box gene family. In core eudicots, two subclades co-exist, \textit{euAG} and \textit{PLE}, as a result of major duplication events early in the history of the core eudicots (Kramer et al., 2004). In Arabidopsis, the C-class gene required for stamen and carpel development and floral meristem determinacy is represented by one \textit{euAG-subclade} gene, \textit{AG (AGAMOUS)}, and loss-of-function mutants of this gene show conversion of stamen and carpel into a petal and a new flower, respectively (Bowman et al., 1991; Yanofsky et al., 1990). In \textit{Antirrhinum majus}, one \textit{PLE-subclade} gene, \textit{PLENA}, has functions as a C-class gene and loss of function results in the phenotype that both stamen and carpel are converted into petaloid organs (Bradley et al., 1993). Arabidopsis has two genes in the \textit{PLE-subclade}, \textit{SHP (SHATTERPROOF) 1 and 2}. In contrast with AG gene, however, SHP genes do not function as a C-class gene. Similarly, in Antirrhinum, the \textit{euAG-subclade} is represented by \textit{FARINELLI}, which has only a minor function as a C-class gene (Davies et al., 1999).

Petunia has two genes belonging to the \textit{AG-clade}: the \textit{euAG-subclade} gene \textit{PETUNIA MADS-BOX GENE3 (pMADS3)} and the \textit{PLENA-subclade} gene \textit{FLORAL BINDING PROTEIN6 (FBP6)} (Angenent et al., 1993, 2009; Tsuchimoto et al., 1993). Silencing of
either pMADS3 or FBP6 resulted in partial loss of stamen identity and slightly altered carpel morphology (Kapoor et al., 2002; Heijmans et al., 2012a). The flowers, however, showed generally mild phenotypes: petaloid tissue was very small and no double flowers with a voluminous appearance were formed. On the other hand, flowers with both pMADS3 and FBP6 silenced exhibited near-complete loss of both stamen and pistil identities (Heijmans et al., 2012a). In the most severely affected lines, anthers and stamen filaments were completely converted into large petaloid tissues in whorl 3, new flowers were formed instead of carpels in whorl 4, and ornamental double flowers were produced. These results indicated clearly that unlike the pronounced subfunctionalization observed in Arabidopsis and Antirrhinum, petunia pMADS3 and FBP6 show redundant functions in specifying the identities of stamens and carpels and in flowering termination. Similar results were obtained in Nicotiana benthamiana (Fourquin and Ferrandiz, 2012): the simultaneous down-regulation of both NbAG and NbSHP genes caused typical loss of C-class gene function phenotypes, suggesting that the redundant function among euAG-subclade genes and PLENA-subclade genes is common in solanaceous species. From the point of view of the commercial importance in petunia, we are particularly interested in not only the mechanism(s) by which the specification of stamen and carpel identities is disturbed but also the process by which petaloid stamens are enlarged and new flowers are generated at inner whorls to result in ornamental double flowers. However, information is scant regarding the process of producing decorative double flowers at the late developmental stage, in contrast to the detailed studies conducted at the initial organ identification stage. Even the involvement of homeotic genes in organ development at the late stage has not been well demonstrated.

The application of forchlorfenuron (CPPU) to torenia (Torenia fournieri) flower bud induced paracorolla development that presumably originated from the stamen stipule (Niki et
al., 2012, 2013; Nishijima, 2012; Nishijima and Shima, 2006). Depending on the timing of
the CPPU application, two types of paracorolla, wide and narrow paracorollas, were induced.
Analysis of floral homeotic gene expression to compare the wide and narrow paracorollas
demonstrated the existence of different expression patterns not only in flower bud at the
paracorolla initiation stage but also in paracorolla itself at the late developmental stage (Niki
et al., 2012). In narrow paracorollas, the expression of C-class genes, TfPLE1 and TfFAR,
was suppressed markedly whereas the expression of one A-class gene, TfSQUA, was not
enhanced. In wide paracorollas, the suppression of C-class gene expression was accompanied
by the enhancement of TfSQUA gene expression. None of the petunia orthologs of
Arabidopsis A-class genes has A-class gene function (Heijmans et al., 2012b) and their
functions in flower development are unknown. We were interested in whether or not a similar
process to that observed in torenia paracorolla is operative in the enlargement of petaloid
stamens in C-class gene suppressed petunia flowers.

Virus-induced gene silencing (VIGS) techniques have emerged as efficient reverse
genetics tools to test gene function (Chen et al., 2004; Godge et al., 2008; Liu et al., 2002).
VIGS is an easy, convenient, and rapid technique; thus, it is useful for the analysis of gene
families that act in a redundant manner and require a large number of silencing transformants.

In this study, to gain a deeper insight into the formation of decorative double flowers,
we attempted to silence two C-class MADS-box genes, pMADS3 and FBP6, individually and
simultaneously using a VIGS system in four petunia cultivars. The results indicated cultivar-
dependent variation in the formation of new flowers in whorl 4 of flowers with both
pMADS3 and FBP6 silenced. Furthermore, we compared large petaloid stamens induced by
silencing both pMADS3 and FBP6 with small petaloid stamens induced by silencing only
pMADS3 through morphological observation and A-class gene expression analysis.
2.2 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’ 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

The tobacco rattle virus (TRV)-based VIGS system that uses the suppression of the anthocyanin pathway via chalcone synthase silencing as the reporter as described in Chapter 1. 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 Smal site of pTRV2 $PhCHS$ vector described in Chapter 1. To generate a construct for silencing both $pMADS3$ and $FBP6$ simultaneously, $pMADS3$ and $FBP6$ fragments were fused and cloned into the Smal site of pTRV2 $PhCHS$ vector.

Agroinoculation of TRV vectors

Virus infection was carried out by means of the *Agrobacterium*-mediated infection of petunias, as described by Chen et al. (2004). *Agrobacterium tumefaciens* (strain EHA105) transformed with pTRV1 and pTRV2 derivatives was prepared. The *Agrobacterium* culture 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.
Young leaves of 3-week-old petunia plants were inoculated with the mixture by using a 1 ml disposable syringe without a needle.

**Measurement cell size in petaloid stamen**

The average sizes of epidermal cells in petaloid tissues were measured as described by Yamada et al. (2009) and Nishijima et al. (2006). Petaloid tissues were fixed with a mixture of formaldehyde: propionic acid: ethanol = 5: 5: 90 and stored at 4 °C. Before examination, the fixed tissues were immersed for 30 min each in 50% and 30% ethanol. The tissues were cut into 2 mm squares and immersed in clearing solution (8 g trichloroacetaldehyde monohydrate, 1 ml glycerol, 2 ml H2O). Adaxial epidermises of the petaloid tissues were observed by the Nomarski differential interference contrast method with an optical microscope (BX-53; Olympus, Tokyo, Japan). The cell numbers of five randomly selected angles were counted and the average cell size was determined by dividing the area of the angle by the cell number.

**Quantitative RT-PCR of C- and A-class MADS-box genes**

Quantitative RT-PCR (qRT-PCR) of C- and A-class MADS-box genes in petals and stamens of WT flowers and petaloid stamens of VIGS-induced flowers was performed as described by Ushijima et al. (2012) and Puerta et al. (2009) with slight modification. Total RNA extraction and contaminated genomic DNA digestion were conducted using a Plant Total RNA Mini Kit according to the manufacturer’s instructions (Favorgen Biotech Co., Ping-Tung, Taiwan). First-strand cDNA was synthesized from 0.5 µg of DNase-treated RNA with ReveTra Ace reverse transcriptase (Toyobo, Osaka, Japan) and random hexamer primer. qRT-PCR was performed with SYBR Premix Ex Taq II (TaKaRa, Ohtsu, Japan) on a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Each reaction was performed with 4 µl of a 1: 20 (v/v) dilution of
the synthesized cDNA with 0.4 µM of each primer in a reaction volume of 20 µl. The cycling conditions were as follows: 95 °C for 2 min followed by 45 cycles of denaturation at 95 °C for 5 s and annealing and extension at 59 °C for 20 s. The specificity of the PCR amplification was verified by dissociation curve analysis. *Elongation factor 1 alpha (EF1-alpha)*, which was found to be the suitable reference gene in petunia (Mallona et al., 2010), was used as the internal control to calculate the efficiency of the cDNA synthesis. The forward primers used for qRT-PCR were designed to span an exon/intron junction to avoid amplification of genomic DNA sequences (*Table 2.1*). Transcript levels of C-class genes (*pMADS3* and *FBP6*) and A-class genes (*FBP29*, *PFG*, and *FBP26*) were normalized to that of *EF1-alpha* and absolutely quantified as described by Ushijima et al. (2012).
Table 2.1 Primers used in this study.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5’ to 3’)*</th>
<th>Usage</th>
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<tr>
<td>pMADS3 fw</td>
<td>TCCCCAGCAGTCATTTCGATG</td>
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<tr>
<td>pMADS3 rv</td>
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</tr>
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<td>RT-FBP29 rv</td>
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* Small letters indicate the intron inserted position.
2.3 Results

*Double flower phenotypes induced by virus-induced silencing of C-class genes in four petunia cultivars*

VIGS treatments of each of the C-class MADS-box genes, pMADS3 and FBP6, and of both pMADS3 and FBP6 simultaneously were conducted in four petunia cultivars, ‘Cutie Blue’, ‘Fantasy Blue’, ‘Picobella Blue’, and ‘Mambo Purple’. 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 ‘Picobella Blue’ and ‘Mambo Purple’, however, no white color were observed, even in flowers with marked homeotic conversion (Fig. 2.1 e, h), 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 double flowers were observed (Figs. 2.1 and 2.2 b), indicating the strong and complete silencing in those cultivars.

In flowers of petunias inoculated with either pMADS3-VIGS or FBP6-VIGS, morphologically significant conversions in whorls 3 and 4 were observed, but only small changes in the commercial appearance were recognized regardless of cultivar (Fig. 2.1a-d). In flowers of pMADS3-VIGS inoculated petunias, the anthers were often converted into small petaloid tissues whereas the stamen filaments retained their original structures (Fig. 2.1 c, d). Carpel tissues were similar to WT although in a few flowers, the apical part of the pistils was slightly cleaved. In flowers of FBP6-VIGS inoculated petunias, the stamens were almost unaffected although very small petal-like tissues arose from connective tissues in a few flowers (data not shown). The carpels exhibited clear conversion although the severity of the aberrations was variable; the stigmas were split in a few flowers and only the apical part of the pistils was cleaved in some flowers (data not shown). In petunias inoculated with
pMADS3/FBP6-VIGS, prominent double flowers with a highly ornamental appearance were formed (Figs. 2.1e, f and 2.2). Complete loss of stamen identity was observed: not only the anthers and but also the stamen filaments were completely converted into petaloid tissues. Moreover, the upper limb-like tissues enlarged significantly in comparison to the small petaloid tissues in pMADS3-VIGS petunias. This complete conversion of stamens into petaloid tissues was observed in all the four cultivars. In whorl 4 of flowers inoculated with pMADS3/FBP6-VIGS, on the other hand, cultivar-dependent conversion of carpels into new flowers was observed. In more than 50% of the double flowers of ‘Mambo Purple’, a second new flower arose instead of a carpel (Fig. 2.2h, i). This process was repeated, generating third and fourth new flowers. Furthermore, in a few flowers, ectopic new flowers emerged from the axil of whorl 3 organs (Fig. 2.3). ‘Mambo Purple’ with both pMADS3 and FBP6 silenced exhibited a voluminous and decorative appearance with a high commercial value. In the other three cultivars, on the other hand, the formation of new flowers in whorl 4 was not recognized in any double flowers in spite of observations of more than 100 double flowers (Figs. 2.1e, f and 2.2a-f). Therefore, it is assumed that the formation of new flowers in whorl 4 by silencing both pMADS3 and FBP6 may be unique to ‘Mambo Purple’.
Fig. 2.1 Morphological changes in flowers of P. hybrida cv ‘Cutie Blue’ inoculated with pTRV2-PhCHS/pMADS3 (pMADS3-VIGS) and pTRV2-PhCHS/pMADS3/FBP6 (pMADS3/FBP6-VIGS). (a) WT flower; (b) stamens and a carpel of WT; (c) pMADS3-VIGS flower (white and blue mixed color); (d) petaloid stamens and a carpel of pMADS3-VIGS flower; (e) pMADS3/FBP6-VIGS flower (white); (f) petaloid stamens and a carpel of pMADS3/FBP6-VIGS flower (white). Scale bars = 1 cm.
Fig. 2.2 Morphological changes in flowers of *P. hybrida* cv ‘Fantasy Blue’, ‘Picobella Blue’, and ‘Mambo Purple’ inoculated with pTRV2-PhCHS/pMADS3/FBP6 (pMADS3/FBP6-VIGS). (a-c) ‘Fantasy Blue’; (d-f) ‘Picobella Blue’; (g-i) ‘Mambo Purple’; (a, d, g) WT flowers; (b, e, h) pMADS3/FBP6-VIGS flowers; (c, f, i) stamens and carpels or converted new flowers of pMADS3/FBP6-VIGS flowers. Scale bars = 1 cm.
Fig. 2.3 New flower formation in whorl 4 and from axil of whorl 3 in a double flower of P. hybrida cv ‘Mambo Purple’ inoculated with pTRV2-PhCHS/pMADS3/FBP6 (pMADS3/FBP6-VIGS). (a) An opened double flower with a second new flower in whorl 4 and an ectopic new flower emerging from the axil of whorl 3; (b) an opened second new flower; (c) fused corolla (left), a carpel (center), and petaloid stamens (right) of the second flower; (d) an ectopic new flower emerging from the axil of whorl 3; (e) an unconverted stamen (left) and petal-like tissues of the ectopic new flower. Other floral organs in the ectopic new flower appeared to be fused to the petal-like tissues and could not be identified (separated) clearly.
To further investigate differences between small and large petaloid stamens, surface areas and average cell sizes of small petaloid stamens in pMADS3-VIGS plants and large petaloid stamens in pMADS3/FBP6-VIGS plants were measured in ‘Cutie Blue’, because many white flowers indicative of complete silencing were produced in this cultivar. The surface areas of petaloid stamens in pMADS3/FBP6-VIGS plants were more than 10 times as large as those in pMADS3-VIGS plants (Fig. 2.4). As the upper limb-like region of the large petaloid stamens in pMADS3/FBP6-VIGS plants accounted for more than 90% of the total area, the size difference was assumed to be mostly due to the development of this region. Then, the upper limb-like region was anatomically compared. The average sizes of epidermal cells in plants inoculated with pMADS3/FBP6-VIGS were only 1.5 times as large as those in plants inoculated with pMADS3-VIGS (Fig. 2.5a). The epidermal cells in either petaloid stamen showed a similar conical shape, which is typical of an epidermal cell of petunia petal (Fig. 2.5b).
Fig. 2.5 Average cell sizes (a) and microscopic angles (b) of adaxial epidermal cells in a petaloid stamen of P. hybrida cv ‘Cutie Blue’ inoculated with pTRV2-PhCHS/pMADS3 (pMADS3-VIGS) and pTRV2-PhCHS/pMADS3/FBP6 (pMADS3/FBP6-VIGS). In (a), the cell numbers of five randomly selected angles per unit petaloid stamen were counted and average cell size was determined by dividing the area of the angle by the cell number. Vertical bars indicate SE (n=6). In (b), scale bars=0.01 µm.
qRT-PCR analysis of C- and A-class MADS-box genes

qRT-PCR analysis of C-class MADS-box genes, pMADS3 and FBP6, indicated that in the small petaloid stamens of pMADS3-VIGS plants, the expression of pMADS3 was suppressed whereas that of FBP6 was slightly increased (Fig. 2.6a). In the large petaloid stamens of plants inoculated with pMADS3/FBP6-VIGS, the expression of both pMADS3 and FBP6 was suppressed significantly. In order to determine whether or not the SQUAMOSA/AP1/FRU type A-class genes are under the regulation of C-class genes and involved in the development of petaloid stamens even at the late developmental stage, the expression of FBP29, PFG, and FBP26 in pMADS3-VIGS and pMADS3/FBP6-VIGS petaloid stamens was analyzed. The expression of FBP29 and PFG was below the detection limit of our system in all the tissues examined, including WT petal (data not shown). FBP26 expression was detected in WT petal, but the expression was very low in stamen (Fig. 2.6b). In the petaloid stamens of plants inoculated with either constructs, FBP26 expression was suppressed compared to the expression in WT petals; however, no significant difference was noted between the small and large petaloid stamens. Together, the results indicate that the expression of SQUAMOSA/AP1/FRU type A-class genes at the late developmental stage has very little relation to petaloid stamens.
Fig. 2.6 Quantitative RT-PCR analysis of C-class MADS-box genes, pMADS3 and FBP6 (a), and A-class MADS-box gene, FBP26 (b), in petaloid stamens of *P. hybrida* cv ‘Cutie Blue’ inoculated with pTRV2-PhCHS/pMADS3 (pMADS3-VIGS) and pTRV2-PhCHS/pMADS3/FBP6 (pMADS3/FBP6-VIGS). The transcript levels of the C-class and A-class genes were absolutely quantified and normalized to that of the EF1-alpha. Vertical bars indicate SE (n=5).
2.4 Discussion

*Cultivar-dependent conversion of carpels into new flowers in whorl 4 in C-class gene silenced petunia*

In view of their commercial value as ornamental plants, double flowers with large petaloid stamens and/or new flowers at inner whorls are desired. To further understand the formation of decorative double flowers with a voluminous appearance in petunia, double flowers induced by VIGS of C-class homeotic genes were investigated. We attempted to visualize silenced flowers/tissues by using tandem VIGS constructs containing *PhCHS* as the reporter and the target C-class gene(s). In ‘Picobella Blue’ and ‘Mambo Purple’, no flowers with even a white spot were observed, in spite of the formation of double flowers (Fig. 2.2e, h). Chen et al. (2004) reported large variations in the generation of white flowers or spots among the cultivars when *PhCHS* was used as the gene-silencing reporter for VIGS in petunia, suggesting a strong influence of genetic background on the gene-silencing phenotype. In ‘Cutie Blue’ and ‘Fantasy Blue’, completely white flowers were produced (Figs. 2.1c-e, 2.2b-c), allowing us to investigate the flowers in which the target C-class gene(s) were strongly and wholly silenced.

It has been demonstrated in petunia that the silencing of either *pMADS3* or *FBP6* induced the partial loss of stamen identity and generated a mild double flower phenotype with small petaloid tissue (Kapoor et al., 2002; Heijmans et al., 2012a), whereas the silencing of both *pMADS3* and *FBP6* led to the near-complete loss of both stamen and pistil identities and, in the most severely affected lines, the complete conversion of anthers and stamen filaments into large petaloid tissues in whorl 3 and carpels into new flowers in whorl 4 (Heijmans et al., 2012a). In this study, we attempted to silence C-class genes using the VIGS technique in four petunia cultivars and obtained similar results for the conversion of stamens in all the
cultivars: flowers of \textit{pMADS3}-VIGS plants bore small petaloid stamens and flowers of \textit{pMADS3/FBP6}-VIGS plants bore large petaloid stamens (Figs. 2.1 and 2.2).

Meanwhile, the new flower formation in whorl 4 of \textit{pMADS3/FBP6}-VIGS plants was observed only in one cultivar. In ‘Cutie Blue’, ‘Fantasy Blue’, and ‘Picobella Blue’, the styles of \textit{pMADS3/FBP6}-VIGS flowers were significantly shorter and thicker than those of WT and the styles and stigmas were transformed into sepal-like organs (Figs. 2.1f and 2.2c, f). In spite of observations of more than 100 double flowers in \textit{pMADS3/FBP6}-VIGS plants, the formation of new flowers was not observed at all in those cultivars, even in the entirely white flowers, and stamens were completely converted into petaloid tissues. In ‘Mambo Purple’, on the other hand, more than 50% of the double flowers in \textit{pMADS3/FBP6}-VIGS plants harbored new flowers in whorl 4 and the formation of ectopic new flowers at the axils of petaloid stamens was observed in a few flowers, producing a highly voluminous appearance (Figs. 2.2h, i, and 2.3). In Heijmans et al. (2012a), flowers in \textit{fhp6/fhp6 pMADS3-RNAi} plants (possessing mutated \textit{fhp6} homologously) showed complete conversion of carpels into secondary flowers, whereas flowers in \textit{fhp6/+ pMADS3-RNAi} plants (possessing mutated \textit{fhp6} heterologously) maintained carpel-like appearances, suggesting the requirement of complete loss of \textit{FBP6} function for new flower formation in whorl 4. In the present study, as VIGS occurred randomly and distinguishing silenced tissues at the organ identification stage from non-silenced tissues was impossible, we could not compare the suppression levels of C-class genes among the cultivars at this stage. Nevertheless, in ‘Fantasy Blue’ and Cutie Blue’, we observed many entirely white flowers, indicating the occurrence of strong and complete silencing, although new flower formation was not found in any of those flowers. Using the VIGS system, we could not exclude the possibility that the difference in frequency of new flower formation among cultivars is a result of a variation in movement of TRV containing
and FBP6 into carpel primordia. Nonetheless, we favor the hypothesis that in addition to the suppression levels of C-class genes, other genetic backgrounds or factors related to the control of flower meristem determinacy, such as the function of D- and E-class MADS-box genes and the petunia WUS homologue, TERMINATOR, participate in the cultivar-dependent difference observed in this study (Angenent et al., 2009; Ferrario et al., 2006). The development of a breeding process that produces stable C-class gene suppressed lines and introduces the genotypes into various cultivars would allow us to prove the hypothesis.

Size of petaloid tissues induced by C-class gene silencing is mostly determined at early developmental stage

To determine the process of generating large petaloid tissues operating downstream of C-class gene silencing, large petaloid stamens induced by pMADS3/FBP6-VIGS were compared with small petaloid stamens induced by pMADS3-VIGS. The size difference was partly attributed to the difference in the effects of the constructs on the identification of stamen tissues, similar to that described in Heijmans et al (2012a). pMADS3-VIGS converted anther but not filament into petaloid tissue, whereas pMADS3/FBP6-VIGS converted both anther and filament. In addition, large differences were observed in the development of the upper limb-like area of petaloid tissues (Fig. 2.1d, f) and thus, the effects of suppressing C-class gene function not only at the organ initiation stage but also at the developmental stage, such as cell division and/or cell enlargement of petaloid tissues, were presumed.

Measurement of cell size in the upper region of petaloid stamens showed only a small difference (1.5 times larger in pMADS3/FBP6-VIGS petaloid tissues; Fig. 2.5) in comparison to the measurement of total surface area (10 times larger; Fig. 2.4), indicating that not cell size but cell number accounted for the total surface area of the petaloid tissues. In developing
petals of petunia, the increase in cell number at the early developmental stage and the increase in cell size at the late developmental stage were reported (Nishijima et al., 2006). It is suggested that petaloid stamen size is mostly determined at the early stage of development.

In Arabidopsis, the enhanced expression of A-class genes (*AP1* and *AP2*) in the inner whorls of AG-suppressed double flower mutants has been reported (Drews et al., 1991; Gustafson-Brown et al., 1994). In CPPU-treated torenia, the generation of wide paracorolla was associated with the enhanced expression of *SQUAMOSA/AP1/FRU* type A-class gene, *TjSQUA*, together with the suppressed expression of C-class genes, both in primordia at the paracorolla initiation stage and in paracorolla themselves at a later developmental stage. In petunia, three genes, *FBP29*, *PFG*, and *FBP26*, that clustered into the *SQUAMOSA/AP1/FRU-clade* have been reported (Immink et al., 2003; Rijpkema et al., 2009). To date, however, neither the expressional regulation of these petunia orthologs by C-class genes in inner whorls nor their function as A-class genes has been reported. In this study, we analyzed the expression of those three genes in the petaloid stamens generated by VIGS of C-class gene(s). The results denoted that the expression levels of those genes were low in all the tissues examined. *FBP26* expression was barely detected in WT petal and very low in stamen (Fig. 2.6b). The *FBP26* expression was also barely detected in petaloid stamen; however, this expression seemed to be a result of homeotic conversion into petal-like tissues, and no difference was observed between small and large petaloid stamens. Thus, the expression of A-class genes in petaloid stamens at the late developmental stage had little biological contribution to petaloid stamen size. Six SQUA-like genes were isolated from Gerbera hybrida and the expression of all the six genes in petal at the early developmental stage was detected by in situ hybridization (Ruokolainen et al., 2010), thereby attracting much attention as regards their functions in petal development. As described above, VIGS
occurred randomly and analysis of A-class genes at the early stage of petaloid stamen development could not be conducted in this study. As reviewed by Heijmans et al. (2012b), data for A-class genes are scant in comparison with those for B- and C-class genes and a systematic comparative analysis of A-class genes in various species is required. The analysis of A-class genes at the early stage of petaloid stamen development using stable C-class gene suppressed petunia lines is expected to improve our understanding of the role of A-class genes in flower development.

2.5 Summary

In conclusion, the results obtained by investigating double flowers induced by silencing C-class genes, pMADS3 and FBP6, showed that 1) new flower formation in the inner whorl of flowers having both pMADS3 and FBP6 silenced is cultivar-dependent, and 2) the size of petaloid tissue induced by silencing C-class gene(s) is mostly determined at the early stage of flower development. These results are valuable for future breeding of petunia cultivars bearing decorative double flowers with a voluminous appearance.
Chapter 3

Application of the VIGS system for functional analysis of genes related to petal senescence Ethylene Insensitive 2 (EIN2) and EIN3 like (EIL) in petunia

3.1 Introduction

Senescence represents the last stage of floral development and is an active process that requires gene transcription and protein translation. A genetically controlled senescence program allows for the ordered degradation of organelles and macromolecules and the remobilization of essential nutrients from the petals. In some floricultural plant including petunia, ethylene is involved on petal senescence. Studies on function ethylene signal during the past decades, progress in understanding the molecular mechanism of ethylene perception and signal transduction has been made through screening for Arabidopsis mutants defective in the ethylene triple response phenotype, and a framework has been established in this model plant. Ethylene is perceived by a family of membrane-associated receptors, which act as negative regulators of ethylene response, along with the downstream Raf-like serine/threonine kinase called CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1). In the absence of ethylene, the active receptors interact with CTR1 to repress the downstream response. While binding ethylene, the receptors are inactive, resulting in the inactivation of CTR1 and the downstream repression is relieved. Then the ethylene signal is transmitted through ETHYLENE-INSENSITIVE 2 (EIN2) into the nucleus to active the EIN3/EIN3-like (EIL) transcription factors, which trigger transcription of downstream target genes, such as ETHYLENE RESPONSE FACTOR 1 (ERF1), ultimately inducing diverse ethylene responses (Guo and Ecker 2004; Chen et al. 2005; Lin et al. 2009).

The transcription factor EIN3, located at the most downstream position of the ethylene signaling pathway, has received more attention than any other components (Chen et
al. 2005). EIN3 functions as a positive regulator of ethylene response (Chao et al. 1997) and a potential integration point for cross-talk with other signals (Yanagisawa et al. 2003; Chen et al. 2005; Zhu et al. 2011). This protein belongs to a small family, of which six members (EIN3 and EIL1 to EIL5) have been identified in Arabidopsis. These members share common features for nuclear-localized transcription factors whereas only EIN3 and its most closely related EIL1 have been conclusively demonstrated to function in the ethylene signaling pathway (Chao et al. 1997; Alonso et al. 2003; Guo and Ecker 2004). To date, the EIN3/EIL gene family has also been well documented in many other plant species including tobacco (Kosugi and Ohashi 2000; Rieu et al. 2003), tomato (Tieman et al. 2001; Yokotani et al. 2003), carnation (Waki et al. 2001; Iordachescu and Verlinden 2005), rice (Mao et al. 2006), banana (Mbeguie et al. 2008) and Oncidium (Chen et al. 2011). These EIN3/EIL genes are almost ubiquitously expressed throughout the plants, despite slightly different patterns and levels of their expression. In Arabidopsis, the regulation of EIN3 at the post-transcriptional level has been reported to be crucial in ethylene signaling. EIN3 mRNA level is not affected by ethylene, however, EIN3 protein is stabilized and accumulates in response to ethylene, while it is rapidly degraded through the 26S proteasome pathway in the absence of ethylene (Guo and Ecker 2003; Yanagisawa et al. 2003). By contrast, Arabidopsis EIL1 shows significant differences in expression upon ethylene treatment (De Paepe et al. 2004; Chen et al. 2005), and EILs in other species such as carnation (Waki et al. 2001; Iordachescu and Verlinden 2005), banana (Mbeguie et al. 2008), and Oncidium (Chen et al. 2011) have also been found to be regulated at the transcriptional level by ethylene and developmental cues.

Petunia, an important ornamental plant, often serves as a model for ethylene-sensitive flower senescence because the plants flower profusely and have large floral organs amenable to molecular and biochemical analysis. Ethylene is clearly involved in modulating the process, but the transcription factors and other components of the senescence signal transduction
pathway(s) remain to be elucidated. Ethylene biosynthesis, perception and signal transduction have become the molecular biology research hotspots in order to extend the vase life and improve the quality of petunia as ornamental flower. Floral senescence in petunia is regulated by the plant hormone ethylene. Ethylene Insensitive 2 (EIN2) and EIN3 like (EIL) are transcription factors functioning for transduction of ethylene signal. The role of petunia Ph-EIN2 was reported by Shibuya et al. (2004). In this study, EIN3 like (EILs) from several species of Solanacea; tomato, tobacco, potato and petunia (Ph-EIL1) nucleotide sequences were collected and created phylogenetic tree (Fig. 3.1). Phylogenetic analysis shows that EILs from several species of Solanacea are separated into 5 clusters. We are planning to clone and silence all of them at once or some of them at once in several combinations to see which EIL genes(s) mainly contribute to petal senescence.

Fig 3.1 Phylogenetic tree of EIN3 like (EIL) from tomato, tobacco, potato and petunia.
3.2 Materials and Methods

*Plant materials and growth conditions*

Petunia (*Petunia hybrida*) seeds of cultivar ‘Cutie Blue’ 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.

*Cloning of PhEIN3 likes (PhEILs)*

EILs from several species of Solanacea; tomato, tobacco, potato and petunia (PhEIL1) nucleotide sequences were collected and created phylogenetic tree. From this phylogenetic tree showed that EILs separated into 5 clusters. Based on this phylogenetic tree, primers were designed (Table 3.1) and full-length cDNAs for *Ph-EIL1, Ph-EIL2, Ph-EIL3, Ph-EIL4* and *Ph-EIL5* were isolated by RT-PCR. For *Ph-EIL2* we used *Nt-EIL5* mRNA nucleotide sequence, *Ph-EIL3* and *Ph-EIL4* we used tomato homolog *Le-EIL2* and *Le-EIL1* nucleotide sequences and for *Ph-EIL5* we used *Nicotiana benthamia* genome sequence to design primers.

Amplified fragment were ligated into pZero vector and finally transformed into E.coli EHA:105(Kyoto) strain. The positive clones identified by PCR analysis and were sequenced using Big Dye ®XTerminator TM Kit with gene-specific primers. Five cDNA fragments of *Ph-EIL1, Ph-EIL2, Ph-EIL3, Ph-EIL4* and *Ph-EIL5* were isolated.

*Plasmid construction EIN2 and EIN3 like (EILs) for VIGS*

At this point, we have cloned 5 types of cDNAs *Ph-EIL1*, Group1 (G1), Group2 (G2), Group3 (G3), and Group4 (G4) encoding *EILs* from petunia petals. I planned to silence all of them at once by using a conserved region of the *EILs*. Therefore, based on 71% to 93% of
conserved region EILs homologous I designed specific primers and amplified the fragment to create a construct that simultaneously silencing the five EILs petunia (Fig 3.3). In addition, there have been reported that silencing of Ph-EIN2 extend flower longevity compared to wild type petunia (Shibuya et al., 2004). Therefore, we create Ph-EIN2 construct as a control for the EILs silencing. A cDNA fragment of petunia chalcone synthase, Ph-CHS, was amplified and cloned into the EcoR1 site of pTRV2 vector to form pTRV2 Ph-CHS as described in chapter 1. Ph-EILs and Ph-EIN2 genes were amplified using the primers listed in Table 2 and cloned into the SmaI site of pTRV2 Ph-CHS vector individually to generate constructs for silencing Ph-EILs and Ph-EIN2 separately (Fig. 3.2). VIGS were conducted as described in Chapter 1.

![Diagram of pTRV2-CHS/EIN2 and pTRV2-CHS/EIN3 like (EILs) vectors](image)

**Fig. 3.2** Vector construct of pTRV2-CHS/EIN2 and pTRV2-CHS/EIN3 like (EILs) for VIGS
**Fig. 3.3** Multiple alignment of the nucleotide sequences from PhEIL1, G1 (PhEIL2), G2 (PhEIL3) and G4 (PhEIL5) genes. Black shading identifies the residues shared by all genes in the group. Conserved region of EILs among all sequences are indicate by black shading. Multiple alignment were done by Genetyx Mac.
**Flower longevity and Propylene treatment**

To measure flower longevity/senescence induced by pollination, we collected WT, CHS, Control (purple flower of VIGS), *EIN2* and *EILs* flowers which divided to two groups pollinate and not-pollinate flowers. For *EILs* VIGS flower we collected only pollinated flower because lack of flower sample.

For propylene treatment (untreated, 100ppm, 150ppm, 200ppm, of 750ppm discontinuous treated propylene) we collected WT and *EIN2* VIGS flowers. The flowers were put in a flask with distilled water (30mL, 50mL) then sealed in a container and placed under fluorescent light at 23°C room. Day which the flowers completely wilted was recorded. Untreated flowers were not put in a sealed container just placed under fluorescent light at 23°C room. Because of the humidity sealed container was high, we using desiccant to control the humidity.

**RNA extraction and quantitative RT-PCR**

Sampling of WT and EIN2 VIGS flower were collected. For EIN2 VIGS sampling, we divided into white and purple petal. For RNA extraction, tissue samples were frozen immediately in liquid nitrogen and stored temporarily at -80°C. Quantitative RT-PCR (qRT-PCR) of EIN2 gene in white and purple petal was performed as described by Ushijima et al. (2012) and Puerta et al. (2009) with slight modification. Total RNA extraction and contaminated genomic DNA digestion were conducted using a Plant RNA Isolation Reagent (Invitrogen) Kit according to the manufacturer’s instructions (Favorgen Biotech Co., Ping-Tung, Taiwan). First-strand cDNA was synthesized from 0.5 µg of DNase-treated RNA (Tri-Reagent RNA clean-Up kit) with ReveTra Ace reverse transcriptase (Toyobo, Osaka, Japan) and random hexamer primer. qRT-PCR was performed with SYBR Premix Ex Taq II
(TaKaRa, Ohtsu, Japan) on a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions. Each reaction was performed with 2 µl of a 1: 10 (v/v) dilution of the synthesized cDNA with 0.2 µM of each primer in a reaction volume of 10 µl. The cycling conditions were as follows: 95 °C for 3 min followed by 45 cycles of denaturation at 95 °C for 5 s and annealing and extension at 59 °C for 30 s. The specificity of the PCR amplification was verified by dissociation curve analysis. *Elongation factor 1 alpha (EF1-alpha)*, which was found to be the suitable reference gene in petunia (Mallona et al., 2010), was used as the internal control to calculate the efficiency of the cDNA synthesis. The forward and reverse primers used for qRT-PCR were designed (Table 3.1).
Table 3.1 Primers used in this study.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5’ to 3’)</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ph-EIL1 Fw</td>
<td>CGATGACATAAGAGGTGGTGAC</td>
<td>Cloning</td>
</tr>
<tr>
<td>Ph-EIL1 Rv</td>
<td>AGTGTGATACAGGTGGATCCTG</td>
<td>Cloning</td>
</tr>
<tr>
<td>Ph-EIL2 Fw</td>
<td>CTCACCTAACATTCTTCTCAAACC</td>
<td>Cloning</td>
</tr>
<tr>
<td>Ph-EIL2 Rv</td>
<td>CCTATATGAAGAGCTGATAGGGTTG</td>
<td>Cloning</td>
</tr>
<tr>
<td>Ph-EIL3 Fw</td>
<td>CCAACTGCGGTAGTTCTTSGTGTC</td>
<td>Cloning</td>
</tr>
<tr>
<td>Ph-EIL3 Rv</td>
<td>CGAGCATCATGATGGTTGTGTG</td>
<td>Cloning</td>
</tr>
<tr>
<td>Ph-EIL4 Fw</td>
<td>GGAGTGCTCTCTCTCTGCAG</td>
<td>Cloning</td>
</tr>
<tr>
<td>Ph-EIL4 Rv</td>
<td>GGTGTGTCTCAGTGACTCTCTGTC</td>
<td>Cloning</td>
</tr>
<tr>
<td>Ph-EIL5 Fw</td>
<td>ATGATGATGTTGAGGAAATGG</td>
<td>Cloning</td>
</tr>
<tr>
<td>Ph-EIL5 Rv</td>
<td>GGCATGGTGTAATGAAGGAGAGGAG</td>
<td>Cloning</td>
</tr>
<tr>
<td>EIN2.VIGS-Fw</td>
<td>GGATCCCTGTGGTCTAGACAG</td>
<td>Construct</td>
</tr>
<tr>
<td>EIN2.VIGS-Rv</td>
<td>GCTTCGGTGATGCTGACATTCCAC</td>
<td>Construct</td>
</tr>
<tr>
<td>EILs.VIGS-Fw</td>
<td>CAAGAGCTGCAGGATACGCACGC</td>
<td>Construct</td>
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<td>EILs.VIGS-Rv</td>
<td>GTGGAAGGAGTGATGAGAGAG</td>
<td>Construct</td>
</tr>
<tr>
<td>EIN2 RealTime Fw</td>
<td>TTGTTCAGCCCCTTTATTGC</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>EIN2 RealTime Rv</td>
<td>TTCCCGGTCAATAATGGGTA</td>
<td>qRT-PCR</td>
</tr>
</tbody>
</table>
3.3 Results and Discussions

Isolation of Ph-EIN3 likes (Ph-EILs) cDNA in Petunia

Ph-EIL1 and Ph-EIL2

Based on the previously reported Ph-EIL nucleotide sequence, we manage to isolate the full length of Ph-EIL cDNA. PhEIL2 sequence with 2.05 kb fragment was isolated. From the deduced amino acid sequence PhEIL2 encoded by 608 amino acids and have 410 bp 5' region to 1.45 kb of EIN3 (pfam04873) fragment domain (Fig. 3.4).

![The nucleotide sequence and amino acid sequence of Ph-EIL2 clone. EIN3 (pfam04873) fragment domain indicated by blue box and primers by pink box.](image-url)
**Ph-EIL3**

About 2.3 kb Ph-EIL3 cDNA fragment was amplified. Sequence analysis showed that amplified fragment was identical with contig sequence that used for primer design. Since we did not read the full length sequence of the amplified fragment we combined another sequence fragment that we read before. Then, in order to avoid frame shift happen when translate amino acids, we modified parts of the sequences based on Nt-EIL1 tobacco amino acid sequence. Deduced amino acid encoded by 617 amino acids and have 236 bp to 937 bp of EIN3 (pfam04873) conserved domain. (Fig. 3.5)

**Fig. 3.5** The nucleotide and amino acid sequence of Ph-EIL3 clone. EIN3 (pfam04873) fragment domain indicated by blue box, primers by pink box and connected nucleotide sequence by red line.
**Ph-EIL4**

For Ph-EIL4 about 2.0 kb cDNA fragment was amplified. Sequence analysis showed that amplified fragment was identical with contig sequence that used for primer design. Since we did not read the full length sequence of the amplified fragment we combined another sequence fragment that we read before. Then, in order to avoid frame shift happen when translate amino acids, we modified parts of the sequences based on NtEIL3 tobacco amino acid sequence. Deduced amino acid encoded by 614 amino acids and have 262 bp to 1092 bp of EIN3 (pfam04873) conserved domain. (Fig. 3.6)

![Fig. 3.6 The nucleotide and amino acid sequence of Ph-EIL4 clone. EIN3 (pfam04873) fragment domain indicated by blue box, primers by pink box and connected nucleotide sequence by red line.](image-url)
Ph-EIL5

For Ph-EIL5 about 2.0 kb cDNA fragment was amplified. Sequence analysis showed that amplified fragment was identical with Nicotiana benthamiana sequence that used for primer design. Since we did not read the full length sequence of the amplified fragment we combined another sequence fragment that we read before. Then, to avoid frame shift happen when translate amino acids, we modified parts of the sequences. Deduced amino acid encoded by 615 amino acids and have 153 bp to 1010 bp of EIN3 super family (cl0483) domain. (Fig. 3.7)

![Diagram of Ph-EIL5 sequence and domain](image)

**Fig. 3.7** The nucleotide and amino acid sequence of Ph-EIL5 clone. EIN3 super family (cl0483) domain indicated by blue box, primers by pink box and connected nucleotide sequence by red line.
**Flower longevity induced by pollination**

I investigate flower longevity of WT, CHS-VIGS, EIN2-VIGS, and EILs-VIGS petunia flower. I divided flower into pollinate and not-pollinate flower. For pollinate flower results showed that average flower longevity for WT is 4.3 days, while EIN2 and EILs silenced flowers around 10 to 15 days, 9 days respectively. This result indicates that flower senescence delayed around 5 to 11 days (Fig. 3.8). As a control, EIN2 silenced purple flower also had better flower longevity.

![Graph](image)

**Fig. 3.8** Flower longevity of pollinate flowers in WT and VIGS plant inoculate with pTRV2-EIN2, pTRV2- EILs in Cutie Blue.

For not-pollinate flower showed that, average flower longevity for WT is 7 days or one week, while EIN2 silenced flowers around 9 to 13 days. This result indicates that flower senescence delayed around 2 to 5 days (Fig.3.9). Similar to pollinate flower, control flower also delay flower senescence.

![Graph](image)

**Fig. 3.9** Flower longevity of not-pollinate flowers in WT and VIGS plant inoculate with pTRV2-EIN2 in Cutie Blue.
**Flower longevity in propylene treatment**

In any concentration of propylene, flower senescence delayed in EIN2 silenced flower compare to WT flower (**Fig. 3.10, Fig. 3.11**). However, flower longevity of EIN2 silenced flower with untreated propylene were longer than flower treated with propylene. This happen because silencing induced in chimeric manner and maybe EIN2 are not completely silencing. In addition, despite using a sealed container containing a desiccant, humidity of untreated flowers were placed slightly higher than humidity in the container, there is a possibility that the senescence occurred not due to ethylene.

![Graph showing flower longevity after propylene treatment by different concentration in WT and EIN2 VIGS flower](image)

**Fig. 3.10** Flower longevity after propylene treatment by different concentration in WT and EIN2 VIGS flower.

![Images showing flower condition over days](image)

**Fig. 3.11** Effect of 750ppm propylene treatment in WT and EIN2-VIGS flower
**Expression of EIN2 silencing flower (blue and white flower)**

The expression level of Ph-EIN2 in white petal of EIN2 silenced flower were 1/5 of WT petals (Fig. 3.12). In addition, even in purple petal, the expression level was lower than the WT petal. Therefore, there is the possibility that silencing of EIN2 is taking place in the part that silencing of CHS is not visualized. This also can be use to explain why control blue flower have more flower longevity than WT flower on tree as mentioned above. Thus, it has been clarified that Ph-EIN2 is functioning as ethylene signalling factor and involved in the petal senescence. This result matched with Shibuya et al (2004). Further, at this time although I was able to investigated flower longevity on trees, pollinated flower, the results showed flower longevity were extended in EILs-VIGS flower, it was suggested that Ph-EILs also may be involved in petal senescence.

![Fig.3.12 Relative expression level of WT, EIN2 blue and white VIGS petal](image)

*Fig.3.12* Relative expression level of WT, EIN2 blue and white VIGS petal
3.4 Summary

In this paper, there have been clarified that five EILs (Ph-EIL1 to Ph-EIL5) exist in petunia. We have cloned 5 types of cDNAs encoding EILs from petunia petals. However, further analysis is needed because full length CDNA for Ph-EIL3, Ph-EIL4 and Ph-EIL5 are not obtained. The flower longevity of petunia flower where Ph-EILs were attempted to silenced. In the future, details about each of isogenes with VIGS and which EIL(s) is involved in the petal senescence are required.
4.1 Introduction

Pollen development in angiosperms is one of the most important processes controlling plant reproduction. At the same time, pollen development also helps increase commercial values by sustaining flower quality and longevity. Many factors are involved in the senescence of cut flowers. Respiration, transpiration, water absorption ability are due to ethylene that accelerated senescence, and also physiological changes based on changes in the hormone such as cytokinin and abscisic acid. The main factor cited in Ichimura et al 2005, mentioned that the phenomenon that occurs within the plant increase in ethylene production. In many type petal flower like carnation and petunia, petal senescence is caused by ethylene generated from the pistil. Further, it is known that petal senescence accelerate by the ethylene production promoted by pollination and fertilization. Therefore, it is believed by controlling the pollen formation and pistil formation, the longevity of flower can be extended. In addition, in the practical commercialization of genetically transgenic pairs must prevent the recombinant gene to diffuse into the environment. Creating recombinant no pollen fertility is demanded in this factor. It also useful for avoiding pollen stains if we can control pollen development.

Molecular genetic studies have revealed that the B and C functions of the well known ABC model together determine the stamen identity, whereas the C function alone specifies the carpel identity (Coen and Meyerowitz, 1991; Ma, 2005). Several genes have been
identified that are required for normal anther development (Ma, 2005). For example, TAPETUM DETERMINANT1 (TPD1) is involved in development of tapetal cells in Arabidopsis anthers (Yang et al., 2003). The tpd1 mutant is unable to form pollen, and contained decreased numbers of tapetal cells and increased numbers of microsporocytes, suggesting that TPD1 is responsible for differentiation of tapetal precursors into mature tapetal cells (Yang et al., 2003). Overexpression of TPD1 results in enlarged tapetal cells and delayed degeneration of the tapetum, suggesting that TPD1 is also involved in maintenance of tapetum (Yang et al., 2005). Overexpression of TPD1 additionally causes carpel cells to continue dividing until maturation of the siliques, resulting in wider siliques than wild-type Arabidopsis (Yang et al., 2005).

Several Arabidopsis thaliana genes crucial for this process have been isolated and characterized: SPL/NZZ (SPOROCYTELESS/NOZZLE), EMS1/EXS (EXCESS MALE SPOROCYTES1/EXTRA SPOROGENOUS CELLS), TPD1 (TAPETUM DETERMINANT1) and SERK1/2 (SOMATIC EMBRYO RECEPTOR KINASE 1/2) (Schieffthaler et al., 1999; Yang et al., 1999; Canales et al., 2002; Zhao et al., 2002; Yang et al., 2003; Albrecht et al., 2005; Colcombet et al., 2005; Yang et al., 2005; Feng and Dickinson, 2010a). ems1/exs and tpd1 mutants and serk1/serk2 double mutants display the same phenotypes of excess PMCs and absence of the TA (or both TA and ML) (Canales et al., 2002; Zhao et al., 2002; Colcombet et al., 2005; Feng and Dickinson, 2010a). These genes are considered to function in the same pathway to regulate cell fate determination. EMS1/EXS, SERK1 and SERK2 encode membrane-localized leucine-rich-repeat receptor-like protein kinases (LRR-RLK), and TPD1 encodes a putative ligand. EMS1/EXS interacts with TPD1 in vitro and in vivo (Yang et al., 2005; Jia et al., 2008). Based on these findings, cell-cell communication has been proposed as essential for anther cell fate determination and
differentiation. It has been speculated that SERK1/2 and EMS1/EXS form heterodimeric receptors (Colcombet et al., 2005), but the ability of TPD1 to bind heterodimers is unknown. The precise timing and cellular location of protein expression of the proposed signaling pathway components are also unclear.

In rice, two TPD1-like genes have been identified; OsTDL1A encoded from one of these genes binds to MSP1 (MULTIPLE SPOROCYTE1), an LRR-RLK (Zhao et al., 2008). Rice MSP1 and TDL1A genes are proposed to be homologs of Arabidopsis EMS1/EXS and TPD1, respectively. Interestingly, there are phenotypic differences between rice and Arabidopsis mutants. In rice msp1 plants, both anther and ovule are affected (Nonomura et al., 2003), whereas no female phenotypes were reported in Arabidopsis. Although rice msp1 anthers are phenotypically similar to Arabidopsis ems1/exs and tpd1 mutants, an RNA interference (RNAi) line directed against the rice TDL1A gene only has ovule defects (Zhao et al., 2008).

In addition these phenotype also occurred in maize where, maize multiple archesporial cells 1 (mac1) mutants share the key phenotype of extra archesporial cells in both ovule and anther (Sheridan et al., 1996; Sheridan et al., 1999). Based on its sequence similarity and its mutant phenotype, maize mac1 is the ortholog of rice TDL1A. MAC1 functions very early in anther development, prior to tapetal formation. These distinctions suggest that the proposed signaling pathway may have species specific characteristics. In recent years, studies of pollen development have contributed greatly to the understanding of phylogenetic relationships, but little has been known about these events in Petunia.

In this chapter, we cloned pollen formation-related genes (TPD1, EMS1) in petunia and identified gene expression pattern in 4 different stages and organs during flower bud development.
4.2 Materials and Methods

4.1. Plant material and growth condition

Petunia (Petunia hybrida) seeds of cultivars ‘Fantasy Blue’ were obtained from Sakata Seed Co. (Yokohama, Japan). Plants were grown in a greenhouse under natural day and night cycles with a day/night temperature regime of 25 °C/20 °C, respectively. Flower buds at various sizes were collected and developing stamens, pistils, petals and sepals were sampled and kept at -80 °C until total RNA was extracted.

4.2. Cloning of Ph-TPD1 full-length cDNA

Total RNA was extracted by the hot borate method (Wan and Wilkins, 1994) from stamens collected from various flower buds (5 mm to 15 mm in length). First strand cDNA was synthesized by Prime Script II 1st strand cDNA Synthesis Kit (Takara, Japan). Using the first strand cDNA as template, fragment and full length coding region of Ph-TPD1 cDNA were amplified by Prime Star GXL (Takara) with Primer A and B, and, C and D, respectively (Table 4.1). The amplified products were cloned in to pZero vector (Invitrogen) and their sequences were determined.

4.3. Determination of Ph-TPD1 and Ph-EMS1 genomic sequence

Genomic DNA was extracted from young leaves of ‘Fantasy blue’. Using the genomic DNA as template, Ph-TPD1 and Ph-EMS1 genomic sequences were amplified by Prime Star GXL (Takara) with Primer C and D, and, E and F, respectively (Table 4.1). The amplified genomic sequences were cloned in to pZero vector (Invitrogen) and their sequences were determined.
4.4. Sequence analysis

Peptide sequences homologous to TPD1 and EMS1 families were collected from protein databases of 13 plants species by blastp program. Databases of these species were download from Emsembl Plants (http://plants.ensembl.org/index.html) and Phytozome (http://www.phytozome.net). Because kinase domain of EMS1 family are highly homologous to that of other kinase family, only extracellular domain were used as query sequence for blast search.

SignalP4.0 and TMHMM2.0 were used to predicted signal peptide and transmembrane domain of the extracted sequences. Mature protein sequences (TPD1 family) or extracellular domain sequences (EMS1 family) were used for the phylogenetic tree construction. The amino acid sequences were aligned by clustal Ω (Sievers et al. 2011). The neighbour-joining method (Saitou and Nei 1987) was used for genealogical reconstruction.

4.5. Quantitative RT-PCR of Ph-TPD1

Quantitative RT-PCR (qRT-PCR) of Ph-TPD1 was performed as described by Ushijima et al. (2012) and Puerta et al. (2009) with slight modification. Total RNA extraction and contaminated genomic DNA digestion were conducted using a Plant Total RNA Mini Kit according to the manufacturer’s instructions (Favorgen Biotech Co., Ping-Tung, Taiwan). First-strand cDNA was synthesized from 0.5 µg of DNase-treated RNA with ReveTra Ace reverse transcriptase (Toyobo, Osaka, Japan) and random hexamer primer. qRT-PCR was performed with SYBR Premix Ex Taq II (TaKaRa, Ohtsu, Japan) on a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Each reaction was performed with 4 µl of a 1: 20 (v/v) dilution of the synthesized cDNA with 0.4 µM of each primer in a reaction volume of 20 µl. The cycling
conditions were as follows: 95 °C for 2 min followed by 45 cycles of denaturation at 95 °C for 5 s and annealing and extension at 59 °C for 20 s. The specificity of the PCR amplification was verified by dissociation curve analysis. Elongation factor 1 alpha (EF1-alpha), which was found to be the suitable reference gene in petunia (Mallona et al., 2010), was used as the internal control to calculate the efficiency of the cDNA synthesis. The forward primers used for qRT-PCR were designed to span an exon/intron junction to avoid amplification of genomic DNA sequences (Table 4.1). Transcript levels of Ph-TPD1 were normalized to that of EF1-alpha and absolutely quantified as described by Ushijima et al. (2012).

Table 4.1 Primers used in this study.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5’ to 3’)</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-TPD1 Fw</td>
<td>ACNHTNCCNARYGGNATNCC</td>
<td>Degenerate</td>
</tr>
<tr>
<td>B-TPD1 Rv</td>
<td>AANGWRTTNGCRTANTGRAA</td>
<td>Degenerate</td>
</tr>
<tr>
<td>C-TPD1 Fw2</td>
<td>ATGAGCTCTCAATCATTGAAACCGG</td>
<td>Cloning</td>
</tr>
<tr>
<td>D-TPD1 Rv2</td>
<td>TCAGCATATAACGGAGGAAC</td>
<td>Cloning</td>
</tr>
<tr>
<td>E-EMS1Fw2</td>
<td>GAGAATCCTAACCCTTTTGCTACATGG</td>
<td>Cloning</td>
</tr>
<tr>
<td>F-EMS1Rv3</td>
<td>CTCTTTTCTGCTGCTTCTATTAACCCA</td>
<td>Cloning</td>
</tr>
<tr>
<td>PhTPD1qtF</td>
<td>AATTTCAAGGCAGAAAGCAAGA</td>
<td>qRT-PCR</td>
</tr>
<tr>
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<td>PhEMS1qtR1</td>
<td>CCGGTATCAACCCAGTAATTTG</td>
<td>qRT-PCR</td>
</tr>
</tbody>
</table>
4.3 Results

2.1. Cloning and characterization of Petunia TAPETUM DETERMINANT 1 (Ph-TPD1) gene

Degenerate primers A and B listed on Table 4.1 were designed corresponding to conserved regions within At-TPD1 and its homologues (TF/L/IPN/SGM/IP for forward primer and FH/QYANS/TF/L for reverse primer). RT-PCR using these primers and 1st strand cDNAs from flower buds of petunia ‘Fantasy Blue’ as template amplified 227bp Ph-TPD1 fragment. The sequence of this fragment was used as the query in blastx search against genomic databases of tomato and Nicotiana benthamiana on SOL Genomics Network (http://solgenomics.net). As results, Solyc03g097530.2.1, designated as Sl-TPD1, and NbS00036826g0004.1, designated as Nb-TPD1, were hit at highest score in tomato and N. benthamiana database, respectively. Primer C and D listed on Table 4.1 were designed based on their sequences to obtain translated region of Ph-TPD1 cDNA and corresponding genomic sequence.

Translated region of Ph-TPD1 cDNA was 519bp encoding 172 amino acids (Fig. 4.1A). SignalP-4.0 software predicts that deduced amino acid sequence of Ph-TPD1 contains cleavable N-terminal signal peptides, which may target the protein into the endoplasmic reticulum for cellular export (Fig. 4.1A lower case). Ph-TPD1 posses all the cysteine residues that are conserved within TPD1 and TDL proteins from various plants. Predicted mature protein of Ph-TPD1 shares 66% and 85% similarity with At-TPD1 and Sl-TPD1, respectively. It also shares 89% similarity with Nb-TPD1; however the Nb-TPD1 CDS reported on SOL Genomics Network, which we used for deducing the Nb-TPD1 amino acid sequence, is predicted CDS from genomic sequence without mRNA information and may contain miss-annotation at exon/ intron prediction.
Fig. 4.1(A) Alignment of the amino acid sequences of TPD1s and TDL1s. The amino acid sequences of TPD1s and TDL1 of petunia, tomato, arabidopsis and rice, were aligned by clustalW. Conserved sites were marked underneath with asterisks. Six conserved cysteine residues were grey-boxed. Signal peptide sequences predicted by SignalP 4.0 are shown in lower case.

Fig. 4.1(B) A phylogenetic tree for TPD1. Sequence information is listed in Table 4.1. Amino acid sequences of the mature proteins were used for alignment and genealogical reconstruction. Numbers in the tree indicate the percentage of 1000 bootstrap replicates in which a group was found (values <50% not shown). “+” indicate the protein that SignalP predicted no signal peptide.
Phylogenic analysis indicates that TPD1 and TDL proteins from various plants are clustered into three branches; TPD1 branch that includes At-TPD1, TDL1A branch that includes Os-TDL1A and Zm-TDL1A (maize MAC1) and TDL1B branch that include Os-TDL1B (Fig. 4.1B). In that phylogenetic tree, Ph-TPD1 is clustered into TPD1 branch, suggesting the close similarity to At-TPD1. Genomic structure of Ph-TPD1 in comparison to those of Sl-TPD1, At-TPD1, At-TDL1A and Os-TDL1B showed that within the sequences of determined, Ph-TPD1 has two introns at corresponding positions to those in Sl-TPD1, At-TPD1, At-TDL1A and Os-TDL1A (Data not shown).

2.2. Cloning and characterization of Petunia Excess Male Sporocytes 1 (Ph-EMS1) gene

Blastx search using amino acid sequences of At-EMS1 N-terminal extracellular domain as the query against genomic databases of tomato and Nicotiana benthamiana on SOL Genomics Network showed hits on Solyc03g026040.1.1, designated as Sl-EMS1.1, and NbS00007719g0005.1, designated as Nt-EMS1, at highest score, respectively. Primer E and F listed on Table 4.1 were designed based on their sequences to obtain Ph-EMS1 partial genomic fragment. Within the genomic fragment amplified, no intron sequence was predicted as reported in the other EMS1s. The fragment was ## bp long encoding 848 amino acids covering N-terminal leucine-rich-repeat (LRR) extracellular domain and transmembrane domain (TM) (Fig. 4.2A). Deduced amino acid sequence of Ph-EMS1 shares 56% and 79% similarity with At-EMS1 and Sl-EMS1. Ph-EMS1 shows especially high similarity in TPD1-binding region with EMS1s from other plant. It also posses LRRs that are conserved in EMS1s from other plant and transmembrane domain next to C-terminal end of LRR domain as reported in the other EMS1s. Phylogenetic analysis shows that Ph-EMS1 is clustered into a clade that also contains At-EMS1 and rice homologue. Os-MSP1 and is distinct from other
membrane-localized leucine-rich-repeat receptor-like protein kinases (LRR-RLKs) with different functions (Fig. 4.2B).

Fig. 4.2(A) Alignment of the amino acid sequences of EMS1s. The TPD1 binding domain of AtEMS1 was underlined. A sequence unit, LxxLxxLxxNxxGxxPxLxx, was repeated in the binding domain (Jia et al. 2008). Sites identical to the consensus sequence are black boxed. Grey boxes indicate the sites that were conserved by only three hydrophobic amino acid residues, Leu, Ile and Val. Transmembrane regions predicted by TMHMM2.0 are boxed.
Fig. 4.2(B) A phylogenetic tree for EMS1. Amino acid sequences of the extracellular domains predicted by SignalP4.0 and TMHMM2.0 were used for alignment and genealogical reconstruction.
2.3. Expression analysis of Ph-TPD1 and Ph-EMS1 during flower development

Quantitative real time analysis of Ph-TPD1 in stamen of flower buds at different developing stages showed that Ph-TPD1 expression could be detected only in the youngest stage (Fig. 4.3A). The levels of Ph-TPD1 expression in this youngest stage were highly fluctuated within replications and more than five times difference was detected. Expression of Ph-EMS1 was detected in stamen at all stages with highest expression at the youngest stage (Fig. 4.3B). Analysis in various tissues of the buds 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.

![Organ specificity of TPD1 and EMS1 gene expression](image)

**Fig. 4.4** Organ specificity of TPD1 and EMS1 gene expression. Gene expression of TPD1 in four floral organs was detected by RT-PCR (a) and quantified by qPCR (b). cDNAs were synthesized from four floral organs at stage 1 (youngest developmental stage). ‘Sp’, ‘Pt’, ‘St’ and ‘Ps’ represent sepal, petal, stamen and pistil, respectively. **EF1a** is an internal control. Gene expression of EMS1 during anther development was detected by RT-PCR (a) and quantified by qPCR (b).
4.4 Discussion

Pollen development is one of the most important physiological even for plants. It’s control in floricultural crops provides us with many benefits not only in the fields of breeding and seeds production but also in the fields of postharvest flower quality, since in many floricultural crops including petunia, pollination is known to enhance ethylene production at style and shorten the longevity of flowers. In this study, as target genes for controlling pollen formation, we cloned homologues of TPD1 and EMS1 from petunia (Ph-TPD1 and Ph-EMS1) and analyzed their expression patterns in developing flower buds. Deduced amino acid sequence of Ph-TPD1 posses the features conserved in TPD1s and TDLs including At-TPD1, Os-TDL1A and Zm-TDL1 (MAC1), such as N-terminal signal peptides and cysteine residues (Fig. 4.1A). It also clustered into TPD1 clade together with At-TPD1 in phylogenetic analysis, suggesting the close similarity between Ph-TPD1 and At-TPD1 (Fig. 4.1B).

EMS1 is one of the membrane-localized leucine-rich-repeat receptor-like protein kinases (LRR-RLKs) consisting in three domains, leucine-rich-repeats (LRR) extracellular domain including a TPD1 binding region, transmembrane domain, and kinase domain. Kinase domain is highly conserved within LRR-RLKs irrespective of their roles on plant development, while the other domains are divergent dependent on their function. In this study, genomic fragment corresponding to the LRR domain and transmembrane domain were cloned from petunia. The deduced amino acid sequence of Ph-EMS1 is clustered into EMS1 clade in phylogenetic trees drawn using the divergent region. Ph-EMS1 posses LRRs and transmembrane domain at identical position to the other EMS1s already reported. It also shows high conservation with other EMS1s in TPD1 binding region. These close similarity of Ph-TPD1 and Ph-EMS1 with TPD1/TDLs and EMS1s suggest that these petunia homologues
may have important function for controlling pollen development as reported in the arabidopis, rice and maize genes (Yang et al 2003, Zhao et al 2008 and Sheridan et al 1999).

This assumption is also supported by expression patterns of these genes in stamen (Fig. 4.3). *Ph-EMS1* was expressed in stamens of all stages analyzed and highest level of expression was detected at the youngest stage. The expression of *Ph-TPD1* in stamen was detected only at the youngest stage. Moreover, the levels of *Ph-TPD1* expression in the youngest stage were highly fluctuated within replications. Although stamens from much smaller buds could not be sampled in this study, it is hypothesized that even the youngest stage in this study is the stage when expression of *Ph-TPD1* is diminishing and hence the expression levels were varied within replications. The fact that expression of both *Ph-TPD1* and *Ph-EMS1* was detected in same tissue, developing stamen at the youngest stage, suggest that *Ph-TPD1* and *Ph-EMS1* cloned in this study interact with each other to regulate early steps of pollen development.

Expression of both *Ph-TPD1* and *Ph-EMS1* was also detected in developing style and petal (Fig. 4.4). In arabidopis *tpd1* and *ems1/ exs* mutants, no female phenotype was observed (Zhao et al., 2002; Yang et al., 2005). On the other hands, defects in ovule development have been reported in rice EMS1 mutant *msp1* and Os-TDL1A suppressed RANi lines (Zhao et al., 2008). In petunia, *Ph-TPD1* and *Ph-EMS1* might have role in ovule development and petal development, though non of the reports shows involvement TPD1/TDL and EMS1 on petal development. Further study that suppresses *Ph-TPD1* and *Ph-EMS1* using VIGS system established in this thesis would help to reveal the all of these genes in flower development.
4.5 Summary

From petunia, homologues of *TAPETUM DETERMINANT 1 (Ph-TPD1)* and *Excess Male Sporocytes 1 (Ph-EMS1)*, both being reported to regulate pollen development, were cloned. Expression analysis of these genes indicates the possibility that these genes interact with each other and regulate early steps of pollen development. It is also implicated that these genes would be involved in development of other tissues such as ovule and petals. Thus, it is suggested that both *Ph-TPD1* and *Ph-EMS1* are important candidate target genes for further study on functional characterization of flower development related genes using the VIGS system.
Conclusions and Prospects

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.

In Chapter 1, VIGS technique using silencing of chalcone synthase (CHS) gene as a reporter focus on characterization of gene families that act in redundant manner that related to flower quality in petunia. The results showed that silencing of chalcone synthase (CHS) gene as a reporter can be used to detect silencing parts in petunia. The differences in silencing patterns also have been observed among five petunia cultivars. ‘Cutie Blue’ was best material for this system. Based on the results in C-class gene silencing efficiency of CHS silencing depends on flower genetic background, temperature and also environmental factors. It was also revealed that VIGS silencing persist in the long term in petunia. In addition, western dot blot analysis to detect TRV viruses can be useful for determining efficiency of VIGS in an early stage developmental of silencing plant. It is valuable method because it is simple, economical, rapid and sensitive. The technique also appears to be a useful and practical diagnostic technique to detect virus infected plant.
In Chapter 2, to gain a deeper insight into the formation of decorative double flowers with a voluminous appearance, double flowers induced by virus-induced gene silencing (VIGS) of two C-class MADS-box genes, pMADS3 and FBP6, were investigated in four cultivars of Petunia hybrida. 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. In whorl 4, 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 whorl 3 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. Quantitative RT-PCR analysis of SQUAMOSA/AP1/FRU type A-class genes, FBP29, PFG, and FBP26, showed no close relationship between the expression of those genes and petaloid stamen size. The results indicate that the suppressed C-class gene function at the late stage of flower development has little influence on the final size of petaloid tissue.

In chapter 3, floral senescence in petunia is regulated by the plant hormone ethylene. Ethylene Insensitive 2 (EIN2) and EIN3 like (EIL) are transcription factors functioning for transduction of ethylene signal. There have been clarified that five EILs (Ph-EIL1 to Ph-EIL5) exist in petunia. We have cloned 5 types of cDNAs encoding EILs from petunia petals. However, further analysis is needed because full length CDNA for Ph-EIL3, Ph-EIL4 and Ph-EIL5 are not obtained. The flower longevity of petunia flower where Ph-EILs were
attempted to silenced. In the future, details about each of isogenes with VIGS and which $EIL(s)$ is involved in the petal senescence are required.

In chapter 4, focusing on pollen formation-related genes TAPETUM DETERMINANT1 (TPD1) and EXCESS MALE SPOROCYTES1 (EMS1) were cloned and their expressions during pollen development were studied. From petunia, homologues of TAPETUM DETERMINANT 1 (Ph-TPD1) and Excess Male Sporocytes 1 (Ph-EMS1), both being reported to regulate pollen development, were cloned. Expression analysis of these genes indicates the possibility that these genes interact with each other and regulate early steps of pollen development. It is also implicated that these genes would be involved in development of other tissues such as ovule and petals. Thus, it is suggested that both Ph-TPD1 and Ph-EMS1 are important candidate target genes for further study on functional characterization of flower development related genes using the VIGS system.

The results described above show that, 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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