MOLECULAR BIOLOGY OF FLOWER DEVELOPMENT IN *VIOLA PUBESCENS*, A SPECIES WITH THE CHASMOGAMOUS-CLEISTOGAMOUS MIXED BREEDING SYSTEM

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Abstract

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MOLECULAR BIOLOGY OF FLOWER DEVELOPMENT IN *VIOLA PUBESCENS*, A SPECIES WITH THE CHASMOGAMOUS-CLEISTOGAMOUS MIXED BREEDING SYSTEM (109 pp.)

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Plants with both open, out-crossing chasmogamous and closed, self-pollinating cleistogamous flowers are distributed over 50 angiosperm families. The chasmogamous-cleistogamous mixed breeding system is a successful reproductive strategy because it can ensure seed output over a range of environmental and pollinator conditions and maintain sexually produced progeny even in the absence of pollination agents. Many ecologists and biologists have conducted research on the mixed breeding system, but the underlying molecular mechanisms remain largely unknown. The research presented here examined the molecular biology of flower development in a widespread North American violet, *Viola pubescens*. The plant growth regulator gibberellic acid (GA) was reported to induce chasmogamous flowers from cleistogamous ones in some species. Two genes, *VGA20ox* and *VGA3ox*, which encode key enzymes (GA20 oxidase and GA3 oxidase) in the biosynthesis of GA, were identified by reverse transcriptase polymerase chain reactions (RT-PCR) from *V. pubescens*. Semi-quantitative RT-PCR indicated that both genes exhibited increased expression in chasmogamous flowers compared to cleistogamous flowers. Exogenous application of GA3, however, did not induce cleistogamous flowers to open. Thus, GA is involved in the mixed breeding system, but

it is not sufficient to convert the closed status of cleistogamous flowers in *V. pubescens*. GA was demonstrated to target the floral meristem identity gene *LEAFY* (*LFY*) in *Arabidopsis*. Two *LFY* orthologs (*VLFY1* and *VLFY2*) were obtained by screening a genomic library of *V. pubescens*. Semi-quantitative RT-PCR suggested the two *LFY* orthologs function together in the early stages of development of both flower types. Analysis of their promoters indicated they might be differentially regulated. The ABCE floral organ identity genes come into play once the floral meristems are determined. Two A class genes (*VAP1* and *VAP2*), two B class genes (*VAP3* and *VPI*), one C class gene (*VAG*) and one E class gene (*VSEP3*) were identified by RT-PCR. The differential expression patterns of the ABCE floral genes between the chasmogamous and cleistogamous flowers were consistent with their morphological differences. Taken together, most of the floral genes examined here in *V. pubescens* were expressed differently between the two flower types, which might be a response to the different environmental conditions under which the two flower types were produced, contributing to the production of both chasmogamous and cleistogamous flowers.

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Chapter 1: INTRODUCTION

Cleistogamy

Flowering is a critical stage in the lives of sexually reproducing angiospermous plants. Not all flowers are open to show the inner floral parts; some flowers remain closed and produce seeds by self-pollination. The report of such closed and self-pollinating flowers can be dated back to 1660, when botanist Hoffman noticed them on his "*Viola Montana ananthos*" (Uphof, 1938). More people documented the occurrences of such closed flowers on some other plants thereafter; however, it was not until 1867 that the term "cleistogamous (closed marriage) flowers" was proposed by Kuhn to describe the flowers that never open and self-pollinate to set seeds (Uphof, 1938; Culley and Klooster, 2007). Actually, many plants with cleistogamous flowers also produce open and potentially out-crossing flowers, which are known as chasmogamous (open marriage) flowers (Uphof, 1938; Lord, 1981; Culley and Klooster, 2007).

Cleistogamous flowers can be found in distantly related flowering plant groups, including some grasses (Poaceae), milkworts (Polygalaceae), touch-me-nots (Balsaminaceae), legumes (Fabaceae) and violets (Violaceae). According to the most recent review on cleistogamy by Cully and Klooster (2007), cleistogamous flowers have been reported in 693 plants, which are distributed over 228 genera and 50 families, with Violaceae, Poaceae, Fabaceae, and Orchidaceae possessing more cleistogamous species than the other families. Cleistogamy appears to have evolved independently approximately 34 to 41 times at the family level (Culley and Klooser, 2007).

In her review about cleistogamy, Lord (1981) proposed four categories of cleistogamy: preanthesis cleistogamy, pseudocleistogamy, complete cleistogamy, and

"true" cleistogamy. Preanthesis cleistogamous flowers carry out self-pollination when they are floral buds, but will eventually open. Examples of preanthesis cleistogamy can be found in cultivated crop plants such as legumes (Lord, 1981). Pseudocleistogamous flowers usually occur when plants are grown in unfavorable environmental conditions such as poor soil nutrition and extreme temperatures (Uphof, 1938; Lord, 1981). The only difference between pseudocleistogamous flowers and chasmogamous flowers of the same species is the lack of anthesis (Lord, 1981; Culley and Klooser, 2007). The divergence between the two flower types occurs quite late in the flower developmental pathway (Culley and Klooster, 2007). In this case, cleistogamous flowers can be viewed as forms of chasmogamous flowers in which sepals and petals are largely arrested in development, while stamens and pistil(s) are reduced yet functional. Species in the complete cleistogamy category produce only cleistogamous flowers. Some orchids and grasses were reported to be completely cleistogamous (Campbell et al., 1983; Catling, 1983; Freudenstein, 1994, 1999). Because complete cleistogamy was often documented in cultivated plants grown in artificial environments, whether the plants could also produce chasmogamous flowers in natural environment requires further investigation (Lord, 1981; Culley and Klooster, 2007). Other species (e.g., plants in the genera *Impatiens* and *Viola*) with cleistogamous flowers are considered having "true" cleistogamy. Their cleistogamous flowers differ from their chasmogamous ones by the early divergence on the floral developmental pathway and the resulting obvious modifications in floral organs (Lord, 1981; Culley and Klooster, 2007). Recently, Culley and Klooster (2007) revised the categories of cleistogamy, and suggested "preanthesis

cleistogamy" be excluded, because the flowers will open in the end and have the potential to be cross-pollinated. Dimorphic cleistogamy, complete cleistogamy and induced cleistogamy are three types of cleistogamy proposed by Culley and Klooster (2007), with dimorphic cleistogamy the same as "true" cleistogamy of previous author, and induced cleistogamy the same as Lord's pseudocleistogamy.

According to Culley and Klooster (2007), nearly 80% (536 out of 693) of species with cleistogamous flowers fall in the dimorphic category. In fact, plant biologists and ecologists are particularly interested in the dimorphic group of plants because these species produce two flower types with distinct morphologies on the same individual. The following sections will focus on the dimorphic cleistogamous species.

Chasmogamous-cleistogamous mixed breeding system

The dimorphic cleistogamous species produce both open, potentially out-crossing chasmogamous flowers and closed, self-pollinating cleistogamous flowers, in some temperate species often doing so at different slightly or non-overlapping seasons. Evolutionary biologists and population ecologists view this mixed breeding system as a highly successful reproductive strategy (Berg and Redbo-Torstensson, 1999; Masuda et al., 2001; Lu, 2002). On one hand, out-crossing of chasmogamous flowers can produce novel genetic combinations to survive in new or fluctuating environment conditions, promote gene flow among different populations, and buffer against inbreeding depression caused by self-pollination. On the other hand, self-pollination of cleistogamous flowers can maintain successful genotypes for survival in relatively stable microhabitats

immediately around the parent plants, eliminate deleterious recessive alleles, and guarantee seed output under unfavorable and extreme environmental conditions because they need less resources to be produced and need no pollinator to be fertilized. Thus, the collaboration of the two flower types ensures seed production and increases genetic diversity of the offspring, which in turn favors survival and evolution of the species.

Morphological differences between chasmogamous and cleistogamous flowers

Cleistogamous flowers and chasmogamous flowers produced on the same individual have different morphological characteristics. The closed cleistogamous flowers are much smaller in size with reductions in different floral organs. Also, cleistogamous flowers lack attractants for pollinators such as nectars and odor (Lord, 1981).

Ritzerow (1908, reviewed by Uphof, 1938) described chasmogamous and cleistogamous flowers of 30 species in detail. She found that a reduced corolla and androecium in cleistogamous flowers were quite common. Usually, petals of cleistogamous flowers are poorly developed or even absent, and they are not colored. Fewer stamens and anther sacs are produced by cleistogamous flowers (Uphof, 1938). On the other hand, the calyx and gynoecium are normally less affected (Uphof, 1938). But for some species (e.g. *Specularia perfoliata* (L.) A. DC.), reductions occur in all floral organs (Trent, 1940).

Divergences in the pollen grains produced by the two flower types were also frequently reported (Uphof, 1938; Lord, 1981). Madge (1929, reviewed by Uphof, 1938)

observed two kinds of pollen grains in *Viola odorata* L. var. *praecox* Gregory in both flower types: ovoid ones and round ridged ones. The ovoid ones were functional in chasmogamous flowers, while in the cleistogamous flowers, the round ridged ones were active. Others reported that pollen grains produced by the cleistogamous flowers usually had a smaller size and a thinner pollen wall (Lee et al., 1979). Whether the morphologically different pollen grains adopt different pollination mechanisms remains unknown.

Developmental divergences between chasmogamous and cleistogamous flowers

For a long time, cleistogamous flowers were viewed as arrested forms of the chasmogamous flowers caused by suppression of growth (Uphof, 1938). This might be true for species in the category of induced cleistogamy / pseudocleistogamy, where anthesis of cleistogamous flowers is inhibited largely by unfavorable environmental conditions. But in the cases of dimorphic cleistogamy, comparative studies in several species clearly indicated that the two flower types diverge early in their developmental pathways; and heterochrony (i. e. alteration in the relative time of onset and rate of development) was suggested to be the developmental mechanism leading to the production of two flower types (Lord, 1979, 1982; Mayers and Lord, 1983a, 1983b; Minter and Lord, 1983b; Porras and Munoz, 2000). By comparing the development of both flower types in *Lamium amplexicaule* L., *Collomia grandiflora* Dougl. ex Lindl., and *Viola odorata*, Lord (1984) concluded that the more morphologically divergent the cleistogamous flowers were from the chasmogamous flowers, the earlier their

developmental pathways had diverged. Among the three species, cleistogamous flowers of *Lamium amplexicaule* exhibit the least modifications and no significant morphological differences are observed in the two flower types until pollen meiosis occurs (Lord, 1982). On the other hand, flower types in *Viola odorata* can be predicted at the inception because the primordia of cleistogamous flowers are much smaller in size (Mayers and Lord, 1983a, 1983b); and the morphological differences between the two flower types of *V. odorata* are more prominent than those of *L. amplexicaule* and *C. grandiflora* (Mayers and Lord, 1984).

Precocious sexual maturation is always associated with cleistogamous flowers. Earlier initiation of meiosis and/or shorter maturation time ensures the cleistogamous flowers achieve sexual maturity when the flowers are very young (Lord, 1979, 1982; Mayers and Lord, 1983a, 1983b; Minter and Lord, 1983b; Porras and Munoz, 2000). For example, pollen meiosis occurs at 2.18 days after cleistogamous flowers are initiated in *Centaurea melitensis* L.; and the flowers need another 5.05 days to reach the stage of anther dehiscence (when the pollen grains are mature and ready to be released); while the chasmogamous flowers start pollen meiosis 12.14 days after initiation and require 12.5 days for the pollen grains to be functional (Porras and Munoz, 2000). Similarly, in *Collomia grandiflora*, pollen meiosis occurs earlier and lasts for a shorter time in the cleistogamous flowers than in the chasmogamous flowers (Lord et al., 1989).

Factors influencing the production of chasmogamous and cleistogamous flowers

Ecological factors

Effects of various ecological factors, including water, light intensity, photoperiod, soil fertility and temperature, on the chasmogamous-cleistogamous mixed breeding system have been extensively studied. For certain species, some environmental clues seem to be more critical than the others. For example, the tropical plant *Ruellia brevifolia* (Pohl) Ezcurra produce chasmogamous flowers during the rainy season and cleistogamous flowers in the dry season (Sigrist and Sazima, 2002). While in the case of *Ceratocapnos heterocarpa* Durieu, cold temperature in the winter promotes the production of cleistogamous flowers, and chasmogamous flowers are produced in the warm spring (Ruiz de Clavijo and Jimenez, 1993). Generally, favorable environmental conditions promote the production of chasmogamous flowers, while cleistogamous flowers are products of unfavorable or even severe environmental conditions. This is not surprising because the big and showy chasmogamous flowers need more investment of resources than the small and inconspicuous cleistogamous ones (Scheon, 1984). An exception was found in *Oxalis montana* Raf. by Jasieniuk and Lechowicz (1987). They observed that cleistogamous flowers increased in populations in the understory of deciduous forests with a diverse herbaceous species, which indicated good environmental conditions; while more chasmogamous flowers were produced in the deeply shaded coniferous wood with a low diversity of understory herbs, which indicated bad environmental conditions (Jasieniuk and Lechowicz, 1987). A possible explanation is that the understory of a deciduous forest is a relatively undisturbed habitat, so self-pollinated progeny of the cleistogamous flowers, with the same genetic information as their parents, can easily survive (Jasieniuk and Lechowicz, 1987).

Plant growth regulators

A few studies during the late 1970s and 1980s tested selected plant growth regulators for their effect on the transition between chasmogamy and cleistogamy. To date only three plant growth regulators have been studied: gibberellic acid (GA), abscisic acid (ABA), and ethylene.

GA is known for its ability to promote elongation. GA plays important roles in many plant developmental processes including flower development. Cleistogamous flowers from hybrids of *Ruellia tweediana* Griseb. and *R. tuberosa* L. open after treatment with GA (Raghuvanshi et al., 1981). Exogenous application of GA also cause opening of cleistogamous flowers in *Lamium amplexicaule* and *Collomia grandiflora* (Lord, 1980; Minter and Lord, 1983a). However, the anthers of GA induced open flowers in *L. amplexicaule* remain as small as those in the closed flowers (Lord, 1980). In addition, the corolla size of GA induced chasmogamous flowers in *C. grandiflora* is intermediate between normal chasmogamous and cleistogamous flowers (Minter and Lord, 1983a). Thus, GA alone does not appear to be enough to produce true chasmogamous flowers, though it does appear to influence flower opening in these species.

In contrast, ABA will inhibit plant growth under unfavorable environmental conditions. Application of ABA can mimic the effect of water stress in *Collomia grandiflora*, producing almost entirely cleistogamous flowers (Minter and Lord, 1983a). Minter and Lord (1983a) suggested that under conditions of water stress, more cleistogamous flowers were the result of an internal increase of ABA.

Ethylene production increased greatly after pollination in both chasmogamous and cleistogamous flowers of *Salpiglossis sinuata* Ruiz & Pav. However, five-day-old cleistogamous flower buds release much more ethylene (about 22 times) than chasmogamous flower buds of the same stage (Lee et al., 1978). Lee et al. (1978) postulated that the large amount of ethylene prevents expansion of the corolla in cleistogamous flowers. In fact, application of ethephone, a compound that releases ethylene, limits corolla development in both types of flowers in *S. sinuata* (Lee et al. 1978).

The effects of plant growth regulators on the production of chasmogamous versus cleistogamous flowers were only tested in a few species. Clearly, more plants should be studied before drawing general conclusions about the relationship between a certain plant growth regulator and the chasmogamous-cleistogamous mixed breeding system.

Genetics

With the advancement of genetics, researchers began to study the genetic basis of the chasmogamous-cleistogamous mixed breeding system. This research was mostly done in crop species because they are easily maintained and manipulated (Gupton and Neas, 1981; Merwine et al., 1981; Ueno and Itoh, 1981; Khattab, 1982; Chhabra and Sethi, 1991; Watanabe et al. 1992; Takahashi et al. 2001).

Although *Nicotiana tabacum* L. normally produces only chasmogamous flowers, mutated individuals produced only cleistogamous flowers providing material to study the genetic control of cleistogamy (Gupton and Neas, 1981). In the amphidiploid plant

Nicotiana tabacum, all of the progeny (or F_1 plants) produced from crossing a wild-type chasmogamous and a mutant cleistogamous individual had open flowers (Gupton and Neas, 1981). The ratio of chasmogamous to cleistogamous progeny in the F_2 generation was approximately 15:1. Plants, producing both chasmogamous and cleistogamous flowers, were obtained from backcrossing the F_1 individuals to the cleistogamous parent with a segregation ratio of 3:1 (chasmogamous: cleistogamous) (Gupton and Neas, 1981). These data indicated that the inheritance of cleistogamy in this species was controlled by two recessive genes (Gupton and Neas, 1981).

Ueno and Itoh (1981) crossed a chasmogamous wheat *Triticum aestivum* variety to a cleistogamous one. The plants of the F_1 generation produced all chasmogamous flowers while in the F_2 generation both chasmogamous and cleistogamous flowers were produced with a segregation ratio of 63:1 (chasmogamous to cleistogamous). In wheat (a hexaploid), three recessive genes seem to determine cleistogamy. Similar breeding studies were carried out in two tretraploid species: *Gossypium hirsutum* L. and *Triticum durum* L. In these species, the ratio of plants that produced chasmogamous or cleistogamous flowers that resulted from crosses and backcrosses suggested that one recessive gene is sufficient to produce cleistogamous individuals (Khattab, 1982; Chhabra and Sethi, 1991). In a similar study with *Sorghum bicolor* (L.) Moench, a diploid crop, Merwine et al. (1981) found that the combination of a recessive character (papery glume) and a dominant character (rolled glume) prevented the normal opening of flowers, giving rise to cleistogamous.

Soybean, *Glycine max* (L.) Merr., is a tetraploid and generally produces both chasmogamous and cleistogamous flowers on the same plant. Takahashi et al. (2001) used a chasmogamous cultivar and another cultivar which produced cleistogamous flowers throughout the blooming period to determine the genetic basis of cleistogamy in that species. Crossing of the two cultivars produced progeny with chasmogamous flowers. The number of plants in the F_2 generation with chasmogamous flowers was three times greater than those producing cleistogamous flowers. Two groups of F_3 generations were obtained from self pollinating F_2 individuals with either chasmogamous or cleistogamous flowers. The segregation of cleistogamous and chasmogamous in the F_3 progeny indicated that at least two genes, one recessive and one dominant, were involved in the determination of cleistogamy in soybean, and the recessive gene was epistatic to the dominant one.

In yet another study, Watanabe et al. (1992) suggested that cleistogamy was dominantly inherited, at least in *Ainsliaea* species. *Ainsliaea apiculata* Sch.-Bip. has both chasmogamous and cleistogamous flowers while *A. faurieana* Beauverd is a chasmogamous species. Natural hybrids between these two species are diploid like their parents and always produced both types of flowers (Watanabe et al., 1992).

Despite all the efforts, no specific gene(s) has been cloned and clearly shown to be responsible for the production of cleistogamous flowers. More detail-oriented research such as quantitative trait locus analysis (a technique used to map regions of the genome that contain genes involved in specifying a quantitative trait) performed in species with well known genetic backgrounds might shed light on this question.

The chasmogamous-cleistogamous mixed breeding system in Violaceae

The genus *Viola* (violet) belongs to the family Violaceae. The genus contains the greatest number of species with the chasmogamous-cleistogamous mixed breeding system, with as many as 80 species reported to produce both flower types (Culley and Klooster, 2007) and the majority of the 525-600 species in the genus inferred to utilize this breeding system (Ballard, pers. comm.).

Morphology

Chasmogamous flowers of *Viola* usually have conspicuous colors such as blue, violet, yellow and many diverse color combinations. The flowers are zygomorphic. Five petals are arranged with two upper, one on each side and one lower. Dark veins on one or more petals are called nectarguides. The lower petal always has a hollow "spur" projecting backwards to the nectar. In some species, hairs occur at the throat of the two side petals (Beattie, 1969). Each of the five fertile stamens has a very short, almost invisible filament, an anther and a dorsal appendage. The dorsal appendages of the stamens cohere tightly to form a hollow cone, surrounding the middle region of the style (Beattie, 1971). The gynoecium is superior, 3-carpellate, and the ovary is unilocular with parietal placentation. Ovules are many. A mucilaginous stigmatic surface can facilitate reception of dry pollen grains (Beattie, 1974).

Cleistogamous flowers of *Viola* are much smaller in size compared to chasmogamous flowers and remain underdeveloped in appearance, especially in the sepals and petals. In some cases, fewer stamens are formed, and consequently fewer

pollen grains produced (Beattie, 1971). The style curls around or is forced around in the young bud, bringing the stigmatic orifice in close proximity to the anthers (Beattie, 1971). However, the sexual organs mature more rapidly in these flowers, and gametes can be produced when the buds are still very young (Mayers and Lord, 1983a), even at 1-2mm long (Harvey Ballard, pers. comm.).

Flower timing and production

Many violets produce chasmogamous flowers and cleistogamous flowers at different times of the year, especially in the temperate regions, e.g., *V. sororia* Willd. in Ohio. Photoperiod, light availability, temperature, plant age and plant growth stage are considered to function in determining flower timing (Trent, 1940; Elisafenko, 2001; Culley, 2002). However, some tropical mountainous species, e.g. *V. grahamii* Benth. in Mexico, produce both types of flowers simultaneously (Cortes-Palomec and Ballard, 2006).

The relative output of chasmogamous flowers and cleistogamous flowers seem to be influenced by environmental conditions, at least in part. For example, decreased light intensity will cause reduction of both types of flowers in *V. mirabilis* L. (Mattila and Salonen, 1995). In addition, the ratio of cleistogamous to chasmogamous flowers is smaller with less light (Mattila and Salonen, 1995). From an ecological and evolutionary perspective, one might also hypothesize that variable environmental conditions from year to year, for instance in a prairie or alpine meadow, would result in a different ratio of chasmogamous to cleistogamous flowers in the same violet individuals. Comparisons of

long term trends in reproductive output in "stable" (e.g. forest) and "dynamic" (e.g. prairie) violet populations would also be informative. However, no studies examining these last two issues are published.

Pollination

Cleistogamous flowers never open until they are ready to disperse seeds. Thus they are obligatory autogamous and display morphological traits typical of self-pollination. In contrast, chasmogamous flowers have showy color and nectar to attract and reward pollinators. Out-crossing is performed by a variety of insects such as bumblebees, wasps and hoverflies. However, selfing of chasmogamous flowers is found in many *Viola* species (Beattie, 1971, 1976; Berg, 2003; Culley, 2002). Certain visitors, with special feeding positions, can transfer pollen to the stigma-opening of the same flower. Geitonogamy and biparental inbreeding are other possible methods of selfing carried out by pollinators with short flight distances (Beattie, 1971, 1976). Culley found that chasmogamous flowers of the stemmed yellow violet *V. pubescens* Aiton can set fruits even in the pollinator-excluded environment. High seed to ovule ratios in chasmogamous flowers of *V. riviniana* Rchb., *V. hirta* L. and *V. mirabilis* are indications of self-pollination in these flowers (Berg, 2003). Allozyme analysis showed that in a population in central Ohio, chasmogamous flowers of *V. pubescens* had a selfing rate of 0.6 in 1996 and 0.07 in 1997 (Culley, 2002).

Seed set and dispersal

For violets, seeds from the two types of flowers are almost identical in morphology. In many species, cleistogamous flowers are the main source of seeds (Beattie, 1969). However, seeds from chasmogamous flowers of *V. pubescens* appear to be twice as abundant as those from cleistogamous flowers (Culley, 2002). In *V. hirta*, if some chasmogamous flowers are left unfertilized, seed set from these flowers will be less. Correspondingly, later in the year, an increase in the production of cleistogamous flowers and seeds occurs. In this way, the plants maintain the annual seed output at a certain level (Redbo-Torstensson and Berg, 1995).

According to the "near and far model" proposed by Schoen and Lloyd (1984), seeds from cleistogamous flowers will stay near to parent plants while seeds from chasmogamous flowers will be dispersed farther away. However, studies of seed dispersal in *V. hondoensis* W.Becker & H.Boissieu and some *Viola* species in West Virginia showed no significant differences in dispersal distance between the two kinds of seeds (Culver and Beattie, 1978; Masuda and Yahara, 1992).

Inbreeding depression

Fitness of seeds from both flower types in some species of *Viola* was compared to study inbreeding depression. In *V. pubescens*, the two types of seeds are not different in mass; emergence rate of the two types of seedlings were also similar (Culley, 2002). Chasmogamous flowers of *V. canadensis* L. do both out-crossing and selfing to produce seeds. Selfed progeny from both chasmogamous and cleistogamous flowers showed comparable performance in terms of survival rates with out-crossed chasmogamous

progeny (Culley, 2000). In *V. hirta*, *V. mirabilis* and *V. riviniana*, seed abortion rate and mortality of seedlings do not differ between chasmogamous and cleistogamous progeny (Berg and Redbo-Torstensson, 1999). At the same time, cleistogamous seedlings are less vulnerable to sibling and interspecific competition than chasmogamous seedlings (Berg and Redbo-Torstensson, 1999). These reports all indicated no disadvantages of selfing in *Viola*.

Population genetics

Culley et al. (Culley and Wolfe, 2001; Culley and Grubb, 2003) have done some population genetic research in violets. Results from both allozymes and inter-simple sequence repeat (a technique usually used to study population genetics by examining polymorphisms of simple sequence repeats or microsatellites) in *V. pubescens* showed high genetic differentiation among six populations of *V. pubescens*. In the long run, populations in smaller fragments will suffer negative effects of population fragmentation, such as genetic drift, and develop reduced levels of genetic variation.

Viola pubescens

My research focused on a widespread North-American violet, *Viola pubescens* (commonly known as yellow-stemmed violet). This species is easily available, produces an abundance of chasmogamous and cleistogamous flowers over the growing season, and is putatively diploid with n=6 chromosomes (Ballard, 1996). *V. pubescens* is a perennial herb, commonly found in the understory of mesic forests in eastern North America. The

plant produces chasmogamous flowers (Fig.1.1A) in the early spring (mid-April to May), when the canopy is open (Culley and Wolfe, 2001). After the forest canopy has fully leafed out, cleistogamous flowers (Fig.1.1B) are produced (May to September) (Culley and Wolfe, 2001; Culley, 2002). The production of the two flower types does not overlap (Culley, 2002).

However, few molecular, cellular and genetic studies have been done on this mixed breeding system. My research focused on these aspects to facilitate our understanding and provide new insights on the evolution and regulation of this mixed breeding system.

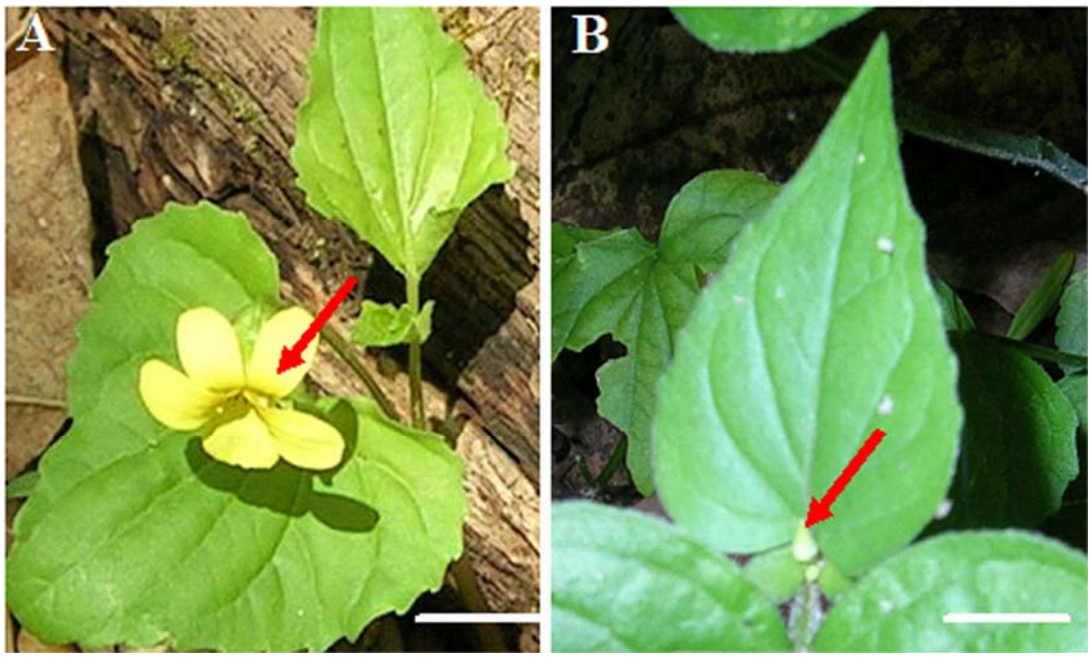

Figure 1.1. Chasmogamous (A) and cleistogamous flowers (B) of *Viola pubescens*. The chasmogamous flowers are open while the cleistogamous flowers are closed. The scale bar equals 1cm.

Molecular biology of flower development

Flowering represents the onset of the reproductive phase. Like other life processes, flowering is controlled by genes. In the past two decades, knowledge of the molecular biology of flowering was greatly improved by the study of model plants such as *Arabidopsis thaliana* (L.) Heynh. and *Antirrhinum majus* L.

Initiation of floral meristem

In *Arabidopsis*, flowering is initiated by four different environmental cues: long-day photoperiod, vernalization (extended cold treatment), autonomous promotion (photoperiod independent pathway), and GA. These environmental conditions indicate cross-talk between flowering pathways that regulate the activities of various downstream genes, which eventually lead to the formation of floral meristems (Fig.1.2, Jack, 2004).

The long-day condition is sensed by some photoreceptors (e.g. PHYTOCHROME A) as well as members of the circadian clock (e.g. EARLY FLOWERING3) (Boss et al., 2004; Jack, 2004). Then a nuclear protein CONSTANS (CO) is activated. The *co* mutants are late flowering in long days, but not in short days (Koornneef et al., 1991). Overexpression of *CO* results in increased expression of *FLOWERING LOCUS T* (*FT*) (Onouchi et al., 2000) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS* (*SOC1*) (Hepworth et al., 2002), suggesting *FT* and *SOC1* are downstream targets of *CO*.

GA is an important stimulator of flowering under short-day condition, because mutants defective in GA biosynthesis (e.g. *ga1* mutant) flower later in short days, but not in long days (Wilson et al., 1992). One target of GA is the floral meristem identity gene

LEAFY (*LFY*), the promoter of which harbors a fragment that responds to GA treatment (Blazque and Weigel, 2000). Moon et al. (2003) reported that the *soc1* null mutant had reduced sensitivity to GA in terms of flowering, thus *SOC1* is another target of GA.

Both vernalization and autonomous promotion pathways work by suppressing *FLOWERING LOCUS C* (*FLC*), a negative regulator of flowering (Boss et al., 2004; Jack, 2004). FLC is an important integrator of flowering signals. Positive regulators of *FLC* include *FRIGIDA* (*FRI*) and *PHOTOPERIOD INDEPENDENT EARLY FLOWERING 1* (*PIE1*). *FRI* keeps plants at vegetative stage until *FLC* is suppressed by factors such as vernalization, while the expression of *FLC* is reduced in *pie1* mutant (Boss et al., 2004; Jack, 2004). In turn, *FLC* suppresses flowering activators *FT* and *SOC1*, which is inferred from the decreased expression of *FT* and *SOC1* in plants overexpressing *FLC* (Hepworth et al., 2002).

Different flowering pathways are converged by floral meristems identity genes including *LFY* and *APETALA1* (*AP1*) (Fig. Fig.1.2, Jack, 2004). *LFY* and *AP1* interact with each other in complicated ways. Overexpression of *LFY* cannot promote flowering in the *ap1* background (Weigel and Nilsson, 1995), while promotion of flowering by 35S:AP1 occurs in the *lfy* mutant (Mandel and Yanofsky, 1995). However, flowers of 35S:AP1 *lfy* have abnormal floral organs (Mandel and Yanofsky, 1995). Also, *AP1* was demonstrated to be a direct target of *LFY* because RNA of *AP1* increases rapidly in the presence of protein synthesis inhibitor when ectopic expression of *LFY* is induced (Wagner et al., 1999). So *AP1* and *LFY* can not act independently of each other in specifying floral meristems.

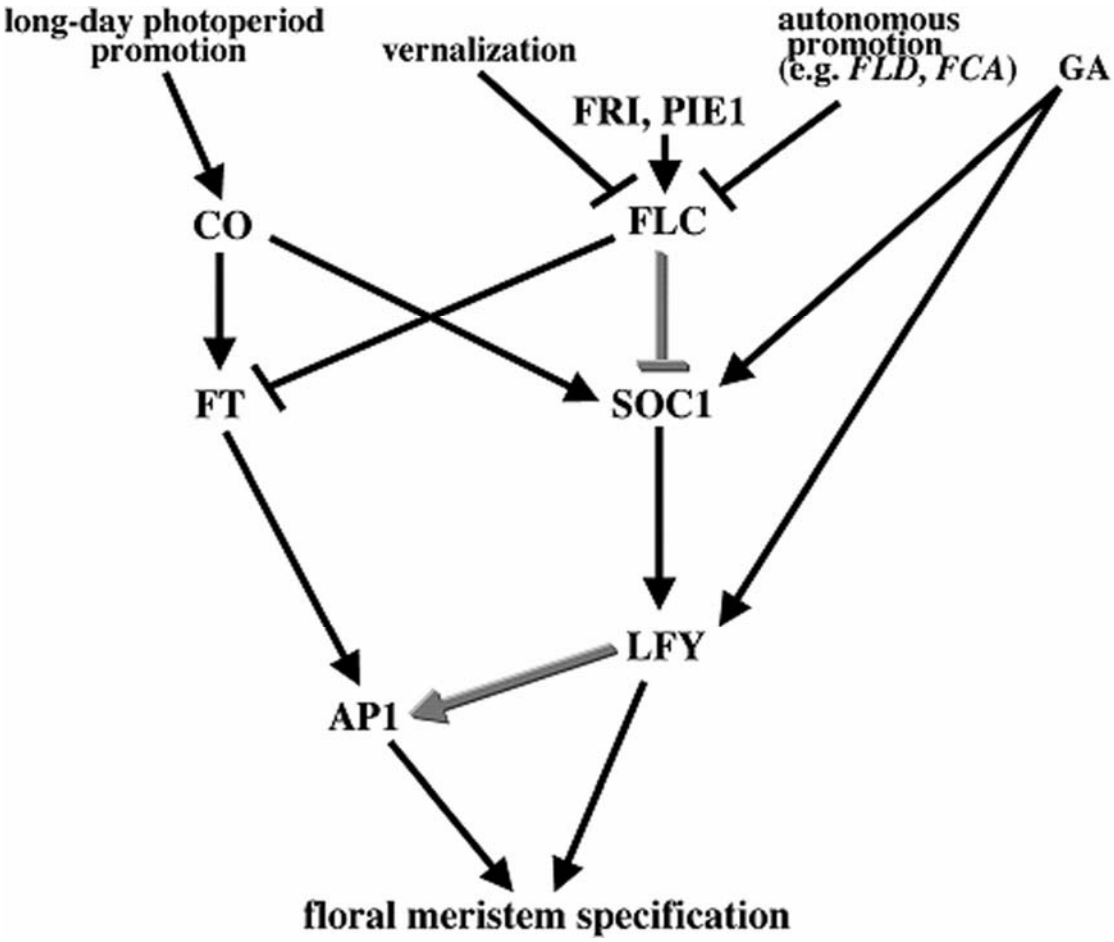

Figure 1.2. Major floral induction pathways in *Arabidopsis*. Flowering in *Arabidopsis* can be induced by long-day photoperiod, vernalization, autonomous promotion and GA. The activation (indicated by →) and repression (indicated by ⊣) of different genes in the pathways lead to the specification of floral meristems. The gray symbols indicate direct regulation. Please refer to the text for detailed explanation. The figure is adapted from Jack, 2004.

Identification of floral organs: from ABC to ABCDE

The differentiation of floral organs follows the formation of floral meristems. A typical flower has four floral organs: sepals, petals, stamens and carpels, which are arranged in concentric rings. Sepals are in the outermost whorl (whorl 1). Petals, stamens and carpels occupy whorl 2, 3 and 4 respectively (Howell, 2000) (Fig. 1.3).

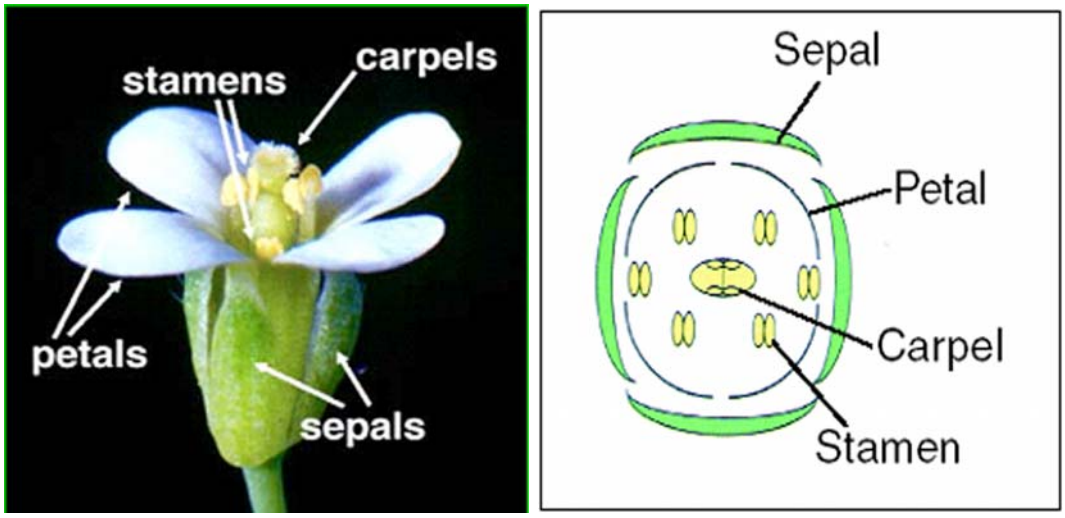

Figure 1.3. Arrangement of different floral organs in an *Arabidopsis* flower. Sepals, petals, stamens and carpels form four concentric rings, with sepals on the outmost whorl. (A). A real *Arabidopsis* flower. (B) A schematic representation of A. The figures are adapted from Howell, 2000.

A set of homeotic genes controls flower development. The "ABC" model is widely accepted to explain how these genes are involved in flowering (Coen and Meyerowitz, 1991, Fig. 1.4A). Three classes of genes, A, B and C, function cooperatively to determine the identity of the four types of floral organs. A class genes give rise to sepals;

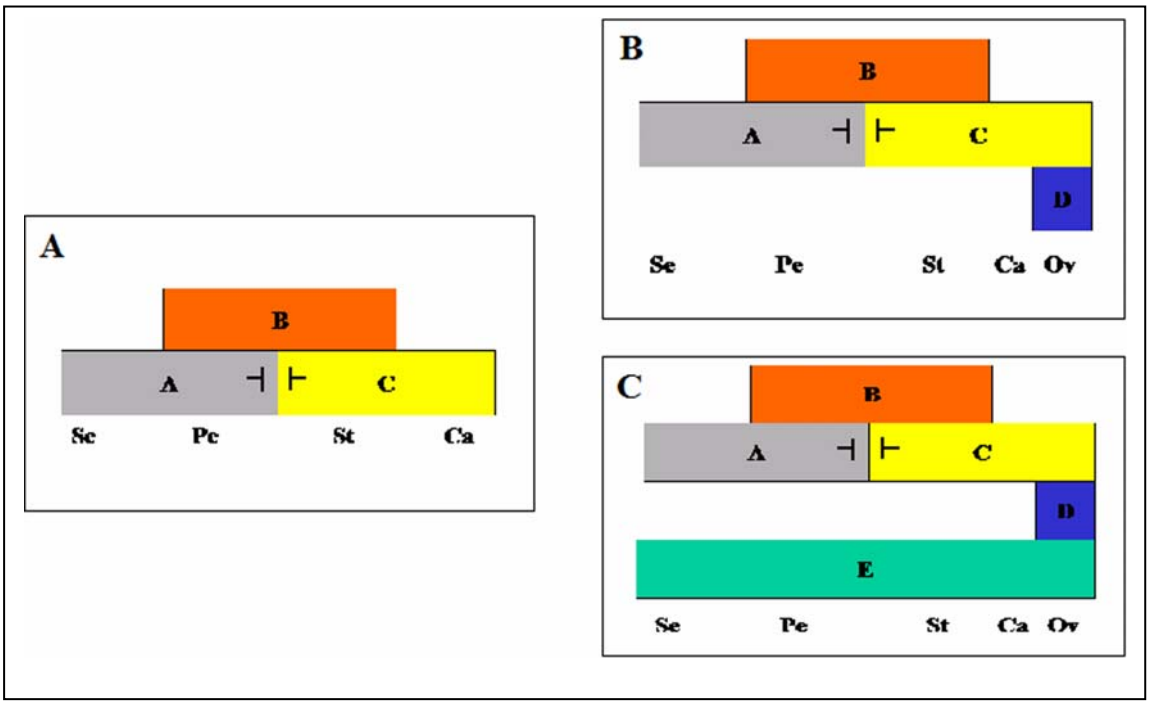

Figure1.4. ABC, ABCD, and ABCDE model of flower development. (A) The ABC model proposes that three classes of genes, A, B, and C, control flower development. Sepals (Se) are controlled by A class genes. Genes in A and B classes specify petals (Pe), while B class genes collaborate with C class genes to specify stamens (St). Carpels (Ca) are initiated by C class genes. The figure was modified after Theissen, 2001. (B) The ABCD model suggests that in addition to the A, B, C classes of genes, D class genes are essential for the development of ovules ovule (Ov). (C) The ABCDE model indicates that the ABCD classes of genes need the cooperation of E class genes to function properly. The figure was modified after Krizek and Fletcher, 2005.

A and B class genes act together to specify petals; B and C class genes are responsible for stamen development, and C class genes alone specify carpels in whorl 4. Furthermore, A and C class genes compete with each other, thus genes in each class work in two adjacent floral whorls (Weigel and Meyerowitz, 1994). Genes in the same class work cooperatively, and mutation of one of them will result in functional loss of that class (Weigel and Meyerowitz, 1994). The ABC model is based on the study of the model plants *Arabidopsis thaliana* and *Antirrhinum majus*, and the "ABC" genes in these two model plants are listed in Table 1.1.

Table1.1

ABC Floral Gene Orthologs in Arabidopsis thaliana *and* Antirrhinum majus

Class	***Arabidopsis thaliana***	***Antirrhinum majus***
A	*APETALA 1(AP1)*	*SQUAMOSA (SQA)*
	APETALA 2(AP2)	
B	*APETALA 3(AP3)*	*DEFICIENS(DEF),*
	PISTILLATA(PI)	*GLOBOSA(GLO)*
C	*AGAMOUS(AG)*	*PLENA(PLE)*

Further research has found that more classes of floral homeotic genes are essential for the development of flowers. Because most known ABC floral genes contain a conserved motif named MADS box, the sequence of MADS box was used to screen a cDNA library generated from the pistils of *Petunia hybrida* Hort. ex Vilm. Two genes, named *Floral Binding Protein 7* and *11* (*FBP7* and *FBP11*), were discovered. Further

studies indicated *FBP7* and *FBP11* were essential for ovule development, and they represented D class genes (Angenent et al., 1995, Fig.1.4B). Later, Pelaz *et al*. (2000) reported that the *Arabidopsis* triple mutant s*ep1sep2sep3* had flowers with only sepals, while flowers of the *sep1sep2sep3sep4* quadruple mutant were like leaves (Ditta *et al.*, 2004). These facts suggested the essential roles of *SEP* genes in flower development, and *SEP1*, *SEP2*, *SEP3*, and *SEP4* are considered E class genes, which are required for the function of the ABC (probably D as well) classes of genes (Pelaz *et al.*, 2000; Ditta *et al*.,2004). The ABC model was extended to ABCDE Model, as is shown in Fig.1.4C (Theissen, 2001, Eckardt, 2003, Krizek and Fletcher, 2005).

The simplicity of the "ABC" ("ABCDE") model has promoted the classification of floral genes in species other than those model plants, providing molecular evidence to study the evolution of plants and flowers. Also, modified ABC models were proposed to describe the development of flowers of non-eudicot plants. Usually these flowers do not have distinct floral whorls and the differences between adjacent floral organs are less obvious. For example, the "Sliding Boundaries" model suggests that the B class gene activity be expanded to sepals or be restricted to stamens, resulting in a petaloid perianth and a sepaloid perianth, respectively (Fig. 1.5B); while the "Fading Border" model was proposed to explain flowers with gradual transitions of organ identities (Fig. 1.5C) (Kramer, *et al*., 2003; Buzgo, *et al*., 2004). In general, the ABCDE model is a landmark in the study of flower development, which integrates floral morphology, molecular biology and evolution.

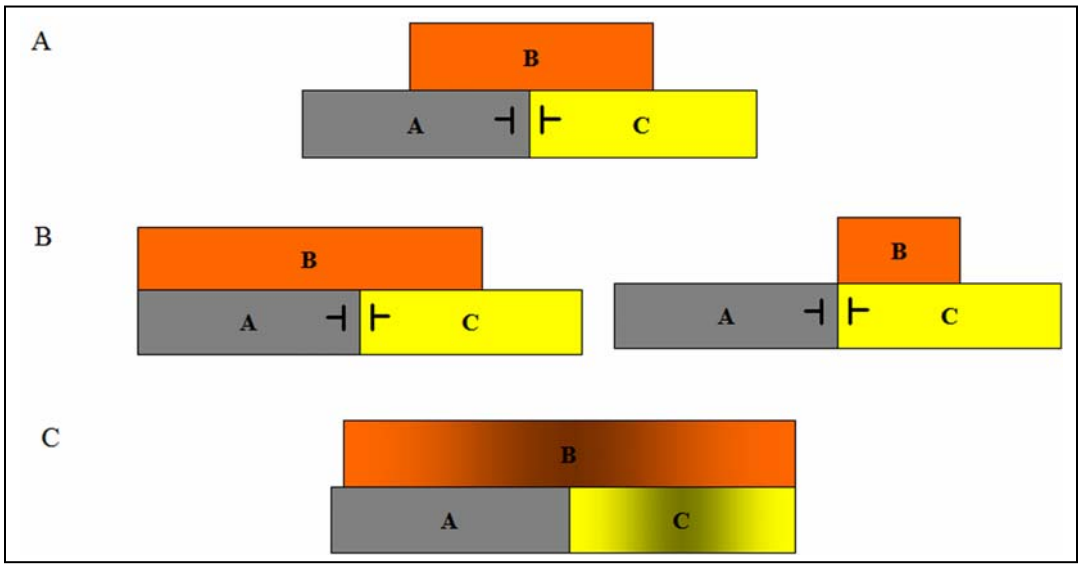

Figure1.5. Modified ABC models to explain flower development in non-eudicot angiosperms. (A) The classical ABC model. The figure was modified after Theissen, 2001. (B) The "Sliding Boundaries" model suggests that the expression of B class genes can be expanded to sepals or restricted to stamens. The expansion of B class genes to sepals explains flowers with petaloid perianth, while the restriction of B class genes to stamens explains flowers with sepaloid perianth. The figure was modified after Kramer, et al., 2003. (C) The "Fading Border" model proposes gradual changes in the expression of all ABC classes of genes, resulting in flowers with gradual transitions in floral organ identities. The figure was constructed after Buzgo, *et al*., 2004.

Significance, specific aims and hypothesis of my dissertation research

My research focused on the molecular mechanisms underlying the chasmogamous-cleistogamous mixed breeding system of a widespread North American violet, *Viola pubescens*. Plants with the chasmogamous-cleistogamous mixed breeding system have breeding advantages; however, as compared to other unusual reproductive strategies (e.g. dioecism), knowledge about the mixed breeding system is sparse. The induction, occurrence, and evolution of the two flower types still remain controversial. Also, no research has explored the molecular basis of this successful breeding system. Studying the chasmogamous-cleistogamous mixed breeding system from a molecular point of view could enhance our understanding of the evolution and maintenance of different plant reproductive mechanisms. Research in this area also has practical applications. For example, if cleistogamous flowers are introduced into genetically modified plants, unexpected gene-flow (gene contamination) from these plants could be restricted. On the other hand, unattractive cleistogamous flowers produced by some gardening plants (e.g. some violets and some orchids) could to be avoided by genetic engineering.

The developmental and morphological differences between the chasmogamous and cleistogamous flowers in *Viola* lead to the hypothesis that there will be a differiential heterochronic expression of the floral genes. For example, earlier initiation of the floral genes, especially of the C class genes that are involved in floral reproductive organ development, is proposed to account for the developmental divergence between the two flower types. But to test this hypothesis, I first need to:

I. Identify genes involved in the flower development of *V. pubescens*

II. Study the expression of the identified floral genes in *V. pubescens* and compare their expression between the two flower types.

Chapter 2: ROLES OF GIBBERELLIC ACID IN THE CHASMOGAMOUS-CLEISTOGAMOUS MIXED BREEDING SYSTEM OF *VIOLA PUBESCENS*

Introduction

Many plants, including most species in the genus *Viola* (Violet), are capable of producing both chasmogamous and cleistogamous flowers (Lord, 1981). The chasmogamous flowers are open with a showy appearance and the potential for out-crossing, while the cleistogamous flowers are closed with underdeveloped floral parts and are self-pollinating. The chasmogamous-cleistogamous mixed breeding system produces seeds of different genetic backgrounds, representing an evolutionarily successful reproductive strategy (Lord, 1981; Lu, 2002). Various environmental factors, such as nutrition availability (Le Corf, 1993), water supply (Wilken, 1982), light intensity and photoperiod (Mayers and Lord, 1983b; Le Corf 1993) have been reported to influence the type of flowers produced by these plants. During the late 1970s and 1980s, some plant growth regulators, including gibberellic acid (GA), were also tested in relation to flower production in several plants. Generally, GA favors the production of chasmogamous flowers (Lord, 1980; Raghuvanshi et al., 1981; Minter and Lord, 1983a). Hybrids of *Ruellia tweediana* Griseb. and *R. tuberosa* L., which only produce cleistogamous flowers, can have chasmogamous flowers by spraying the plants with GA3 (Raghuvanshi et al., 1981). Exogenous application of GA3 also induces the opening of cleistogamous flowers in *Lamium amplexicaule* L. and *Collomia grandiflora* Dougl.ex Lindl. (Lord, 1980; Minter and Lord, 1983a).

GAs are a group of tetracyclic diterpenoid plant growth regulators that participate in many plant growth and development processes including flower development. In *Arabidopsis thaliana* (L.) Heynh., the *LEAFY* gene is essential for the formation of the

floral meristem, and its promoter contains a region that is responsible for GA-induced expression (Blazque and Weigel, 2000). Some floral homeotic genes are also regulated by GA (Okamuro et al., 1997; Yu et al., 2004). Thus, GA might be involved in flower development by mediating the expression of various floral genes.

GA metabolism is altered by light in complex ways that are not well understood. Red light enhances the expression of a GA20 oxidase in pea (Ait-Ali et al. 1998), but the expression a GA20 oxidase in germinating lettuce seeds is inhibited by red light (Toyomasu et al., 1998). GA20 oxidase mRNAs of spinach and *Arabidopsis* increases after long day treatment (Wu et al., 1996; Xu et al., 1997). Symons and Gregory (2003) observed significant decrease of GA in pea during the de-etiolation process.

Viola pubescens Aiton is a widespread North American plant in the genus *Viola* (commonly known as violet), which is famous for the large number of species with the chasmogamous-cleistogamous mixed breeding system. Like many other violets, chasmogamous flowers of *V. pubescens* are produced in the spring, when the forest canopy is open; while cleistogamous flowers appear in the summer, when the canopy has closed, limiting light availability to the understory of the forest (Culley 2002). Because the combinatorial effect of photoperiod and light availability affect the light quality and quantity perceived by the plant, it was hypothesized that the change in light led to differential levels of GAs, which in turn results in the production of the two flower types. To test this hypothesis, orthologs of a GA20 oxidase gene and a GA3 oxidase gene of *V. pubescens* were identified, and their expression compared between the two flower types. Additionally, GA3 was applied to cleistogamous flowers of *V. pubescens* during field

trials. This study provided the first molecular evidence of the relationship between GA and the chasmogamous-cleistogamous mixed breeding system.

Materials and Methods

Plant materials

Chasmogamous and cleistogamous flowers of *V. pubescens* at different developmental stages (from visible buds to mature flowers), as well as leaves, were collected from The Ridges Land Laboratory of Ohio University. Plant materials were preserved in RNAlater RNA Stabilizing Reagent (Qiagen, Valencia, California, USA) immediately after collection.

RT-PCR and semi-quantitative RT-PCR

Total RNA was extracted from flowers and leaves with the RNeasy Plant Mini Kit (Qiagen, Valencia, California, USA) and treated with DNase (Qiagen, Valencia, California, USA). RT-PCR was conducted using the OneStep RT-PCR Kit (Qiagen, Valencia, California, USA). The primers were designed from the conserved regions of GA20 oxidase and GA3 oxidase genes of other plants. Sequences of the primers were as the following: GA20F2: C C (A/C/G) (A/G/T/C) (A/C) (A/G/T) C A (A/G) T T C (A/G) T (A/G/T/C) T G G C C (A/T/G/C) G A; GA20R1: G (A/T) (A/C/T) A G (A/T) G C C A T G A A (G/T) G T G T C (A/G) C C; GA3F2: G A (C/G) C A (C/T) (G/T) (C/T) (C/T) C G (C/T) C A A C T (C/T) T G G; and GA3R3: C A C T (A/C) G G G T A (C/T) (A/C) (A/G) C C C (A/G) T T T G. The RT-PCR program included a reverse transcription step at 50ºC for 30min followed by a step to inactivate the reverse transcriptase at 94ºC for

15min, and then 35 cycles of 30s at 94ºC for denaturation, 30s at 55 ºC for annealing and 1min at 72ºC for extension. Negative controls without reverse transcriptase were conducted to evaluate the possibility of genomic DNA contamination. RT-PCR products were cloned into the TOPO TA cloning vector (Invitrogen, Carlsbad, California, USA) and sequenced on an ABI 310 genetic analyzer. Sequences were analyzed with the Basic Local Alignment Search Tool (BLAST) provided by NCBI to confirm their identities. Alignment of the protein sequences was done by T-Coffee. For the semi-quantitative RT-PCR, the *26S rRNA* gene of *V. pubescens* was used as a control. Products of the semi-quantitative RT-PCR were analyzed on 1.5% agarose gels by electrophoresis. Relative abundance of each floral gene mRNA was normalized to that of the control gene using Image J software (http://rsb.info.nih.gov/ij/).

Exogenous application of GA3

Three plots of *V. pubescens* at the Strouds Run State Park of Athens, Ohio were chosen for this study. The three plots were of similar environmental conditions, and each had more than 100 individual *V. pubescens* plants. Treatment began at the end of May, after the production of chasmogamous flowers had ceased and prior to the production of cleistogamous flowers. One plot was sprayed with 100mg/L GA3 (Sigma, St. Louis, Missouri, USA) + 50mg/L Triton X-100 (Fisher, Pittsburgh, PA, USA). Another plot was treated with 50mg/L Triton X-100 only, while a third plot received no spray treatment. The spray treatment was continued weekly for two months. Plants were monitored twice a week during the treatment. A subset of plants (about 15) from each

plot was harvested at the end of each month and the flowers were examined. Data were analyzed by ANOVA analysis.

Results and Discussion

Identification and characterization of VGA20ox *and* VGA3ox *in* V. pubescens

A 757bp and a 375bp RT-PCR product were obtained from *V. pubescens* with degenerate primers specific for GA20 oxidase and GA3 oxidase respectively. The protein sequences deduced from the nucleotide sequences of the RT-PCR products shared high similarities with the GA20 oxidases and GA3 oxidases in other plant species such as poplar and tobacco (Fig. 2.1). The genes were named *VGA20ox* and *VGA3ox*, with V standing for *Viola*. The expression of *VGA20ox* and *VGA3ox* were then studied in the flowers and leaves of *V. pubescens* by semi-quantitative RT-PCR. For both genes, increased expression was observed in chasmogamous flowers and the leaves subtending them, as compared to cleistogamous flowers and their subtending leaves (Fig. 2.2). This increased expression may be the result of the changing light environment, which, in turn, contributed to the open status of the chasmogamous flowers. Chasmogamous and cleistogamous flowers of *V. pubescens* occur under different photoperiod (short day vs. long day) and shading (open canopy vs. closed canopy) conditions. *VGA20ox and VGA3ox* might respond differently to the varied light conditions, as do the GA-biosynthesis enzymes of other plants (Wu et al., 1996; Xu et al., 1997; Toyomasu et al., 1998). The short photoperiod and/or the open canopy may upregulate the expression *VGA20ox* and *VGA3ox*.

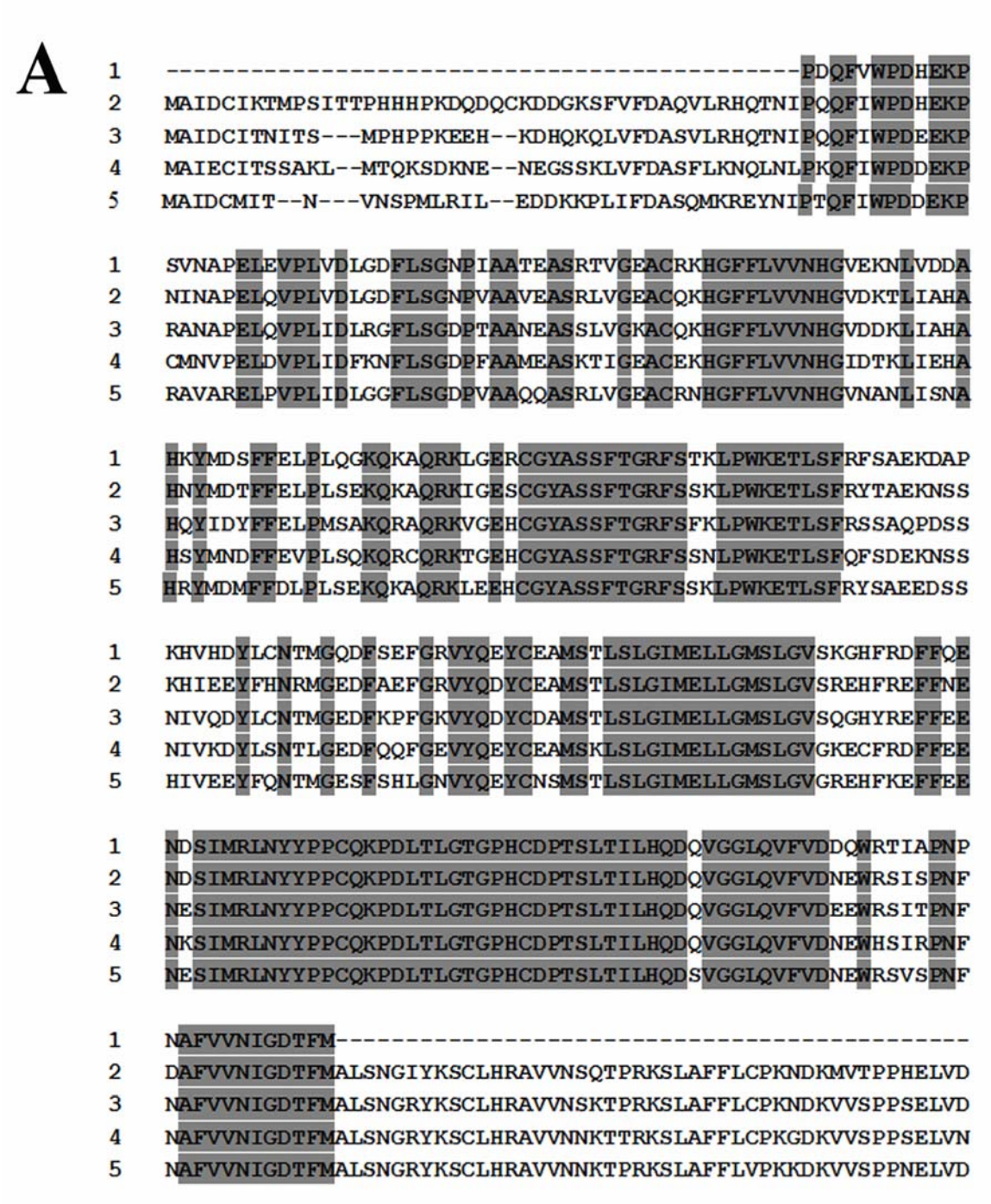
A
1 ----------PDQFVWPDHEKP
2 MAIDCIKTMPSITTPHHHPKDQDQCKDDGKSFVFDAQVLRHQTNIPQQFIWPDHEKP
3 MAIDCITNITS---MPHPPKEEH--KDHQKQLVFDASVLRHQTNIPQQFIWPDEEKP
4 MAIECITSSAKL--MTQKSDKNE--NEGSSKLVFDASFLKNQLNLPKQFIWPDDEKP
5 MAIDCMIT--N---VNSPMLRIL--EDDKKPLIFDASQMKREYNIPTQFIWPDDEKP
1 SVNAPELEVPLVDLGDFLSGNPIAATEASRTVGEACRKHGFFLVVNHGVEKNLVDDA
2 NINAPELQVPLVDLGDFLSGNPVAAVEASRLVGEACQKHGFFLVVNHGVDKTLIAHA
3 RANAPELQVPLIDLRGFLSGDPTAANEASSLVGKACQKHGFFLVVNHGVDDKLIAHA
4 CMNVPELDVPLIDFKNFLSGDPFAAMEASKTIGEACEKHGFFLVVNHGIDTKLIEHA
5 RAVARELPVPLIDLGGFLSGDPVAAQQASRLVGEACRNHGFFLVVNHGVNANLISNA
1 HKYMDSFFELPLQGKQKAQRKLGERCGYASSFTGRFSTKLPWKETLSFRFSAEKDAP
2 HNYMDTFFELPLSEKQKAQRKIGESCGYASSFTGRFSSKLPWKETLSFRYTAEKNSS
3 HQYIDYFFELPMSAKQRAQRKVGEHCGYASSFTGRFSFKLPWKETLSFRSSAQPDSS
4 HSYMNDFFEVPLSQKQRCQRKTGEHCGYASSFTGRFSSNLPWKETLSFQFSDEKNSS
5 HRYMDMFFDLPLSEKQKAQRKLEEHCGYASSFTGRFSSKLPWKETLSFRYSAEEDSS
1 KHVHDYLCNTMGQDFSEFGRVYQEYCEAMSTLSLGIMELLGMSLGVSKGHFRDFFQE
2 KHIEEYFHNRMGEDFAEFGRVYQDYCEAMSTLSLGIMELLGMSLGVSREHFREFFNE
3 NIVQDYLCNTMGEDFKPFGKVYQDYCDAMSTLSLGIMELLGMSLGVSQGHYREFFEE
4 NIVKDYLSNTLGEDFQQFGEVYQEYCEAMSKLSLGIMELLGMSLGVGKECFRDFFEE
5 HIVEEYFQNTMGESFSHLGNVYQEYCNSMSTLSLGIMELLGMSLGVGREHFKEFFEE
1 NDSIMRLNYYPPCQKPDLTLGTGPHCDPTSLTILHQDQVGGLQVFVDDQWRTIAPNP
2 NDSIMRLNYYPPCQKPDLTLGTGPHCDPTSLTILHQDQVGGLQVFVDNEWRSISPNF
3 NESIMRLNYYPPCQKPDLTLGTGPHCDPTSLTILHQDQVGGLQVFVDEEWRSITPNF
4 NKSIMRLNYYPPCQKPDLTLGTGPHCDPTSLTILHQDQVGGLQVFVDNEWHSIRPNF
5 NESIMRLNYYPPCQKPDLTLGTGPHCDPTSLTILHQDSVGGLQVFVDNEWRSVSPNF
1 NAFVVNIGDTFM----------
2 DAFVVNIGDTFMALSNGIYKSCLHRAVVNSQTPRKSLAFFLCPKNDKMVTPPHELVD
3 NAFVVNIGDTFMALSNGRYKSCLHRAVVNSKTPRKSLAFFLCPKNDKVVSPPSELVD
4 NAFVVNIGDTFMALSNGRYKSCLHRAVVNNKTTRKSLAFFLCPKGDKVVSPPSELVN
5 NAFVVNIGDTFMALSNGRYKSCLHRAVVNNKTPRKSLAFFLVPKKDKVVSPPNELVD

Figure 2.1. Alignments of the protein sequences deduced from the nucleotide sequences of the RT-PCR products obtained with primers specific for GA20 oxidase and GA3 oxidase from *V. pubescens* with GA20 and GA3 oxidases in other plant species. Amino acids that are identical for all species were shaded in grey. (A) Alignment for GA20 oxidase. 1. *Viola pubescens*; 2. *Populus nigra*; 3. *Fagus sylvatica*; 4. *Pisum sativum*; 5. *Nicotiana tabacum* L. (B) Alignment for GA3 oxidases. 1. *Viola pubescens*; 2. *Populus tremula x Populus tremuloides*; 3. *Prunus subhirtella*; 4. *Solanum lycopersicum*; 5. *Nicotiana tabacum*.

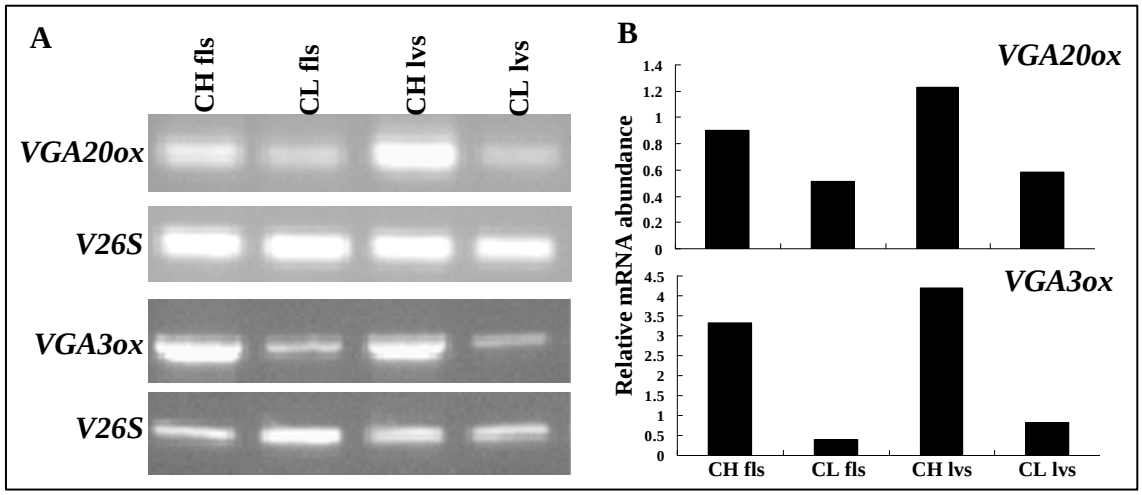

Figure 2.2. Expression of *VGA20ox* and *VGA3ox* in *V. pubescens*. RT-PCR products were obtained from RNA extracted from the flowers and leaves of *V. pubescens* with primers specific for GA20 oxidase gene and GA3 oxidase gene respectively. The 26S rRNA of *V. pubescens* was used as the control. (A) Comparison of the expression of *VGA20ox* and *VGA3ox* between chasmogamous flowers/leaves and cleistogamous flowers/leaves of *V. pubescens*. (B) Relative abundances of the mRNAs of *VGA20ox* and *VGA3ox* to the mRNA of *V26S* in the chasmogamous flowers/leaves and cleistogamous flowers/leaves of *V. pubescens*. CH fls: chasmogamous flowers; CL fls: cleistogamous flowers; CH lvs: leaves subtending chasmogamous flowers; CL lvs: leaves subtending cleistogamous flowers.

The increased expression of the two genes in chasmogamous flowers met expectations, because GA was reported to increase or induce the production of chasmogamous flowers in quite a few species (Lord, 1980; Raghuvanshi et al., 1981, Minter and Lord, 1983a). GA has been shown to regulate the expression of some floral genes in *Arabidopsis*, including *LEAFY*, *APETELA3* and *PISTILLATA* (Okamuro et al., 1997; Blazque and Weigel, 2000; Yu et al., 2004); thus the varied GA levels in *V. pubescens* might cause differential expression of the downstream floral genes, resulting in two flower types. On the other hand, GA is well known for its ability to promote cell division and expansion. Cleistogamous flowers have arrested floral organs, especially in the perianth. For example, smaller cell numbers and reduced cell sizes were documented in the petals of the cleistogamous flowers in *Collomia grandiflora* (Minter and Lord, 1983a). The reduced GA in the cleistogamous flowers might function by limiting cell expansion and division, and eventually prevent the opening of flowers.

Effect of exogenous GA3 on inducing the opening of cleistogamous flowers in V. pubescens

V. pubescens produces chasmogamous flowers at different times with no overlap (Cully 2002). Application of GA3 (100mg/L) was initiated after the production of chasmogamous flowers had ceased and before the occurrence of cleistogamous flowers. Although GA3 treatment was continued weekly for two months, all flowers observed were cleistogamous. However, plants receiving GA3 treatment produced significantly more cleistogamous flowers (about two more flowers per plant) than those treated with Triton X-100 alone or those left untreated (Table 2.1). There was no significant

difference between Triton X-100 treated plants and those without treatment in terms of flower number, indicating the extra flowers produced were an effect of GA3 (Table 2.1).

Table 2.1.

Effect of Exogenous GA3 on the Production of Cleistogamous Flowers in V. pubescens

Treatment Group	Occurrence of chasmogamous flowers	First Harvest		Second Harvest	
		No. of Plants	No. of flowers	No. of Plants	No. of flowers
GA3 + Triton X-100	No	15	4.53 ± 2.03*	17	3.76 ±2.31*
Triton X-100	No	13	2.54 ± 1.66	14	1.71 ±1.20
Control	No	18	2.61 ± 1.15	14	1.93 ± 1.00

* Significant difference at 0.001 levels.

GA3 was reported to turn cleistogamous flowers into chasmogamous flowers in the hybrids of *Ruellia tweediana* and *R. tuberosa* (Raghuvanshi et al., 1981), *Lamium amplexicaule* (Lord, 1980) and *Collomia grandiflora* (Minter and Lord, 1983a), but it failed in the case of *V. pubescens*. However, in species where GA3 has worked, the anthers of the induced open flowers in *L. amplexicaule* remained as small as those of the closed flowers, and the corolla size of the induced chasmogamous flowers in *C. grandiflora* was intermediate in morphology between the normal chasmogamous and cleistogamous flowers (Lord, 1980; Minter and Lord, 1983a). Thus, GA3 alone does not account for all the differences between chasmogamous and cleistogamous flowers. On the other hand, ontogeny studies indicated that chasmogamous and cleistogamous flowers of *Viola odorata* were divergent at inception (the floral meristems of chasmogamous

flowers are bigger than those of the cleistogamous ones), while those of *L. amplexicaule* and *C. grandiflora* were indistinguishable (Mayers and Lord, 1983a). Flower developmental processes of *Viola* may be less flexible than that of *Lamium* and *Collomia*, and the interchange between chasmogamous and cleistogamous flowers may involve additional factors.

In summary, the relationship between GA and the chasmogamous-cleistogamous breeding system in *V. pubescens* was examined. Although both *VGA20ox* and *VGA3ox* showed greater expression in the chasmogamous flowers than the cleistogamous flowers, exogenous GA3 did not change the identity of the cleistogamous flowers. Thus, GA may be involved with, but by itself is not sufficient to trigger, the transition between cleistogamous and chasmogamous flowers in *V. pubescens*.

Chapter 3: IDENTIFICATION AND CHARACTERIZATION OF THE *LEAFY* ORTHOLOG IN A CLEISTOGAMOUS VIOLET, *VIOLA PUBESCENS*

Introduction

The *LEAFY* (*LFY*) gene of *Arabidopsis thaliana* (L.) Heynh. is crucial for the initiation of floral meristems (Weigel et al, 1992; Shannon and Meekswagner, 1993; Weigel and Nilsson, 1995; Wagner et al., 2004). The inflorescence of the *lfy* mutant failed to differentiate into flowers (Schultz and Haughn, 1991), while over-expression of *LFY* induced precocious flowering in *Arabidopsis* (Weigel and Nilsson, 1995; Wager et al., 2004). Furthermore, aspen and citrus plants with over-expressed *LFY* gene could bypass their long juvenile growth and quickly transition to the reproductive phase (Weigel and Nilsson, 1995; Pena et al., 2001). The *LFY* orthologs in diverse plants such as pines, gnetum, maize, grapes, and tobacco were also identified. Molecular and morphological analysis indicated that these *LFY* orthologs function in the transition from vegetative growth to reproductive growth in all species studied (Kelly et al., 1995; Mouradov et al., 1998; Shindo et al., 2001; Carmona et al., 2002; Bomblies et al., 2003; Dornelas and Rodriguez, 2005).

The *LFY* gene encodes a transcription factor and regulates a variety of genes involved in flower development, including the ABC floral organ identity genes (Busch et al., 1999; Wagner et al., 1999; Lamb et al., 2002; William et al., 2004). *APETALA1* (*AP1*) is an A-class gene, functioning in the development of sepals and petals (Coen and Meyerowitz, 1991; Theissen, 2001), and it also contributes to the formation of floral meristems (Mandel, et al., 1992; Ferrandiz et al., 2000). Both functions of *AP1* depend on LFY (Wager et al., 1999; William, 2004). LFY regulated the expression of the B-class gene *APETALA3* (*AP3*) by various ways: it could bind directly to the upstream

regulatory region of *AP3* to promote its expression; alternatively, it could activate *AP3* indirectly with the help of various intermediate molecules (Lamb et al., 2002). Positive regulation of another B-class gene *PISTILLATA* (*PI*) by LFY was also reported (Weigel and Meyerowitz, 1993; Parcy et al., 1998). The second intron of the C class gene *AGAMOUS* (*AG*) harbors a binding site for LFY, and the normal expression of *AG* requires LFY expression (Busch et al., 1999; Hong et al., 2003). In addition, *LFY* orthologs in tomato, petunia, poplar and the grape cv. Riesling were expressed in developing floral organs, clearly indicating their roles in floral organ development (Souer et al., 1998; Molinero-Rosales, 1999; Rottmann et al., 2000; Joly et al., 2004).

Plants that can produce both open (chasmogamous) and closed (cleistogamous) flowers on the same individual occur in 50 angiosperm families (Culley and Klooster, 2007). Chasmogamous flowers are showy and potentially out-crossing, while cleistogamous flowers are rudimentary in morphology, with some organs appearing arrested developmentally, yet sexually functional and produce seeds by self-pollination. The mixed chasmogamous-cleistogamous breeding system ensures seed production over a wide range of environmental conditions, guaranteeing sexually produced progeny even in the absence of pollination vectors, and represents an evolutionarily successful reproductive strategy (Uphof, 1938; Lord, 1981; Lu, 2002). Scientists have studied plants having the mixed breeding system for a long time and have conducted diverse investigations on the morphology, ecology and population biology of the system (Beattie, 1971; Mayers and Lord, 1983a, 1983b; Masuda and Yahara, 1992; Culley, 2001, 2002).

The underlying molecular mechanism of the mixed breeding system, however, is largely unknown.

Because the *LFY* gene and its orthologs are master regulators of both floral meristem initiation and subsequent flower development, the *LFY* ortholog of a violet with the mixed breeding system, *Viola pubescens* Aiton (stemmed yellow violet) was studied, in an effort to understand whether *LFY* plays a role in the differentiation of the two flower types. *Viola pubescens* is a widespread North American violet belonging to a genus famous for the large number of species with the chasmogamous-cleistogamous mixed breeding system (Lord, 1981). Two *LFY* orthologs were cloned from *V. pubescens*, and their expression was compared in different plant organs and at different flower developmental stages to gain insight into the relationship between the *LFY* orthologs and the chasmogamous-cleistogamous mixed breeding system.

Materials and Methods

Plant materials

Plant materials were collected from the Ridges Land Laboratory of Ohio University. Leaves for genomic DNA extraction were collected and stored immediately at -80ºC. Chasmogamous and cleistogamous flowers of *V. pubescens* at different developmental stages (from visible buds to mature flowers), as well as leaves and stems, were collected and preserved in RNAlater RNA Stabilizing Reagent (Qiagen, Valencia, California, USA) immediately after collection.

PCR and sequencing

Genomic DNA extraction and subsequent PCR were conducted using the Extract-N-Amp™ Plant PCR kit (Sigma, St. Louis, Missouri, USA) according to the protocol suggested by the manufacturer. The primers LFYF and LFYR were designed from the conserved regions of the known *LFY* orthologs in other plant species and the sequences can be found in Table 3.1. The PCR products were cloned into the TOPO TA cloning vector (Invitrogen, Carlsbad, California, USA) and then sequenced by an ABI 310 genetic analyzer. The sequence was analyzed with the Basic Local Alignment Search Tool (BLAST) provided by NCBI.

RT-PCR

Total RNA was extracted from flowers and leaves with the RNeasy Plant Mini Kit (Qiagen, Valencia, California, USA) and treated with DNase (Qiagen, Valencia, California, USA). First strand cDNA was synthesized by using the Affinity script QPCR cDNA synthesis kit (Stratagene, La Jolla, CA). The cDNAs were used as templates for subsequent PCR using the Platinum PCR SuperMix High Fidelity (Invitrogen, Carlsbad, California, USA). The sequences of the primers can be found in Table 3.1. Negative controls without reverse transcription were conducted to evaluate the possibility of genomic DNA contamination. The *26S rRNA* gene of *V. pubescens* was used as a control gene. Products of the semi-quantitative RT-PCR were analyzed on 1.5% agarose gels by electrophoresis.

Table 3.1.

Sequences of Primers Used to Study LFY *Orthologs of* V. pubescens

Primer name	***Primer sequence***
LFYF	5'-ACATGA(G/A)(G/A)GACGAGGAG-3'
LFYR	5'-CCAGGCTC(T/C)GT(C/T)ACGAT-3'
VLFYF0	5'-GATGATGAACAGCTTGTCTCAG-3'
VLFYR0	5'-CAGGCTCTGTCACAATAAATGG- 3'
VLFYFS	5'-GCTCTTTCCCAAGAAGGGC-3'
VLFYPSR1	5'-TGCTCGCGCTGTCTCTCGG-3'
VLFYPSR2	5'-TGCTCGCGCTGTCTCTCGC 3'

Southern blotting

Genomic DNA was extracted as previously described by Stockinger *et al*. (1996). Eight restriction enzymes *BamH*I, *Dra*I, *EcoR*I, *EcoR*V, *Hind*III, *Nco*I, *Pst*I, *Xho*I (New England Biolabs, Ipswich, Massachusetts) were used to digest 5μg of genomic DNA respectively. The digested DNA was separated on a 0.8% agarose gel and transferred to a Hybond N membrane (GE Healthcare, Quebec, Canada). A plasmid containing a PCR product from *V. pubescens* with primers specific for *LFY* was used to generate ^{32}P labeled probes by using the High Prime Labeling Kit (Roche Biochemicals, Indianapolis, Indiana). The hybridized membrane was scanned by a phosphor scanner (GE Healthcare, Quebec, Canada)

Genomic DNA construction, screening and sequence analysis

Genomic DNA was extracted as previously described by Stockinger *et al*.(1996). The genomic DNA was partially digested by *Sau*3AI and the fragments between 9-23kb were extracted from low melting point gel by using β-Agarase. The DNA fragments

were ligated into the *λFIXII* vector and the recombinants were added to the packaging extract (Stratagene, La Jolla, CA) to generate a genomic library. The probes described above were used to screen the library. The inserts of the positive clones were then subcloned into pGEM11Z vectors for efficient amplification. After that, the inserts were digested with *Not*I and hydrosheared into 1.0-2.5kb fragments. The 1.0-2.5kb fragments were than subcloned into vector pUC119. Positive clones were sequenced by ABI 310 genetic analyzer and aligned by Vector-NTI to get the final sequences. Promoter analysis was done by Tfsitescan program (http://www.ifti.org/cgi-bin/ifti/Tfsitescan.pl). Nucleotide and protein alignments were performed using the BLAST program provided by NCBI and the T-COFFEE program (http://www.ch.embnet.org/).

Results

V. pubescens *had at least two* LFY *orthologs*

Genomic DNA of individual *V. pubescens* plants was used in the polymerase chain reaction (PCR) with degenerate primers specifically designed for *LFY* (LFYF and LFYR, Table 3.1), and two PCR-products of distinct sizes were detected (Fig. 3.1A). The nucleotide sequences of the two PCR products were of high similarity, and nucleotide-nucleotide BLAST analysis indicated that they shared identities with the *LFY* orthologs in other plant species such as poplar and pea. In addition, the genomic DNA was digested with various restriction enzymes and a Southern Blot was performed. The occurrence of multiple bands in each lane provided another piece of evidence for the existence of more than one copy of *LFY* ortholog in *V. pubescens* (Fig. 3.1B).

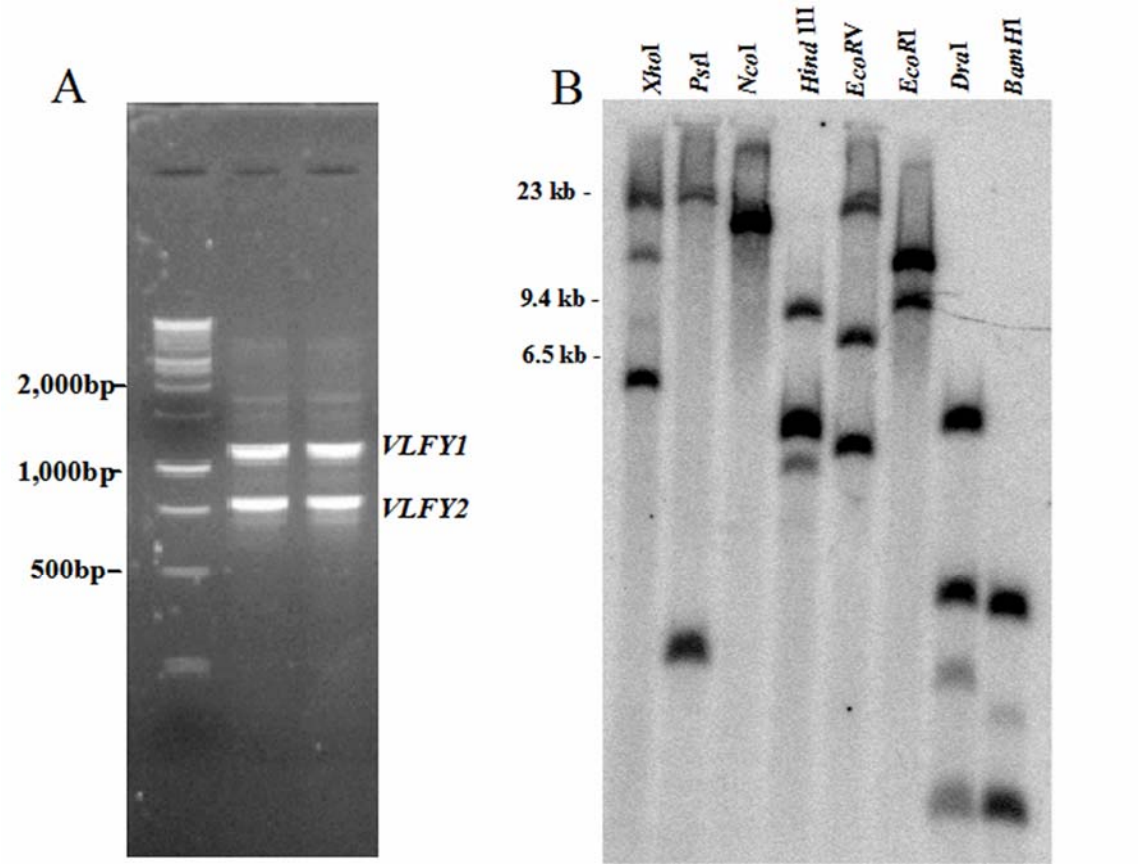

Figure 3.1. Multiple *LFY* orthologs exist in *V. pubescens*. (A) Amplification of *LFY* orthologs from *Viola pubescens* by PCR. PCR was conducted using the genomic DNA from individual *V. pubescens* plants and the degenerate primers specific for *LFY* orthologs. The sequences of the PCR products at the arrows showed homology to *LFY* orthologs in other plant species. (B) Southern Blot for the *LFY* orthologs in *V. pubescens*. Genomic DNA of *V. pubescens* was digested with restriction enzymes *BamH*I, *Dra*I, *EcoR*I, *EcoR*V, *Hind*III, *Nco*I, *Pst*I, *Xho*I respectively and hybridized with probes prepared from the short PCR product in Fig.3.1A.

A genomic library was then constructed and screened with ^{32}P-labled probes generated from the short PCR product mentioned above. Two *LFY* orthologs, named *VLFY1* and *VLFY2*, were discovered. Both *VLFY1* and *VLFY2* were predicted to have three exons and two introns, a typical gene structure of many *LFY* orthologs (Fig. 3.2). The corresponding exons of both genes were of the same size with a few nucleotide differences, while the corresponding introns were quite different. For example, the first intron of *VLFY1* was 711bp, while the one of *VLFY2* was 346bp (Fig. 3.2A). The predicted cDNAs of both *VLFY1* and *VLFY2* encoded proteins of 397 amino acids, 98% of which were identical. Both proteins had the conserved LFY domain, and shared high identities with LFY-like proteins in other plants (Fig. 3.2B).

A 155bp fragment directly upstream of the start codon was analyzed for *VLFY1* and *VLFY2*, in an attempt to gain some insights on the regulation of them. Only 155bp was used because no more than 155bp upstream of the gene was obtained for *VLFY2*. However, the predicted promoter for the *LFY* gene in *Arabidopsis* is only a100bp fragment upstream of the gene (http://arabidopsis.med.ohio-state.edu/), thus the 155bp promoter regions were expected to provide some useful information. Analysis by the program Tfsitescan indicated that some regulatory elements, such as the BPC1-STK and C1-CS sites, could be found in both promoters (Table 3.2). The two promoters also had some different *cis*-elements. For example, the promoter of *VLFY1* harbored GAMyb and AMYBOX1 sites, both which are involved in gibberellic acid and sugar regulated expression, while the presence of phyA3 site in *VLFY2* indicated that this gene might be photo-regulated (Table 3.2).

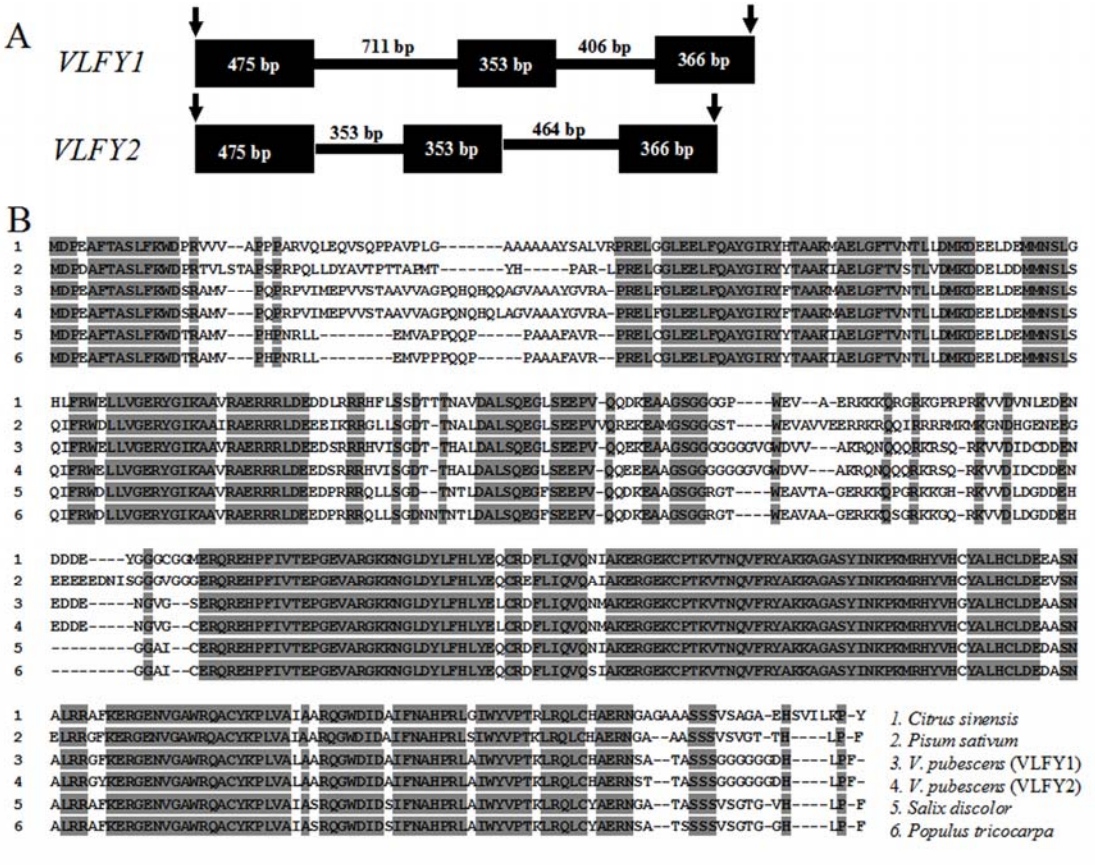

Figure 3.2. Two *LFY* orthologs from *V. pubescens.* (A) Schematic drawing of the gene structures of *VLFY1* and *VLFY2*, two *LFY* orthologs from *V. pubescens*. The boxes represent exons and the lines represent introns. The numbers indicate the molecular size of the introns and exons in base pairs (bp). The arrows indicate the start and stop codons. (B) Alignment of deduced amino acids of VLFY1 and VLFY2 with the LFY-like proteins from other plant species. The identical residuals for all proteins were shaded in grey.

Table 3.2.

Regulatory Elements Found in the Promoters of VLFY1 *and* VLFY2

The symbol "√" indicates the presence of the *cis*-elements in the promoters.

	AMYBOX1	***BPC-STK***	***C1-CS***	***GAMyb***	***PhyA3***	***SEF4***
VLFY1	√	√	√	√		√
VLFY2		√	√		√	

***Expression of* VLFY*s in* Viola pubescens**

Paralog specific reverse transcription PCR (RT-PCR) was used to study the expression of *VLFY1* and *VLFY2* because the high degree of similarity between them made Northern Blot not possible. A set of primers were designed by aligning the sequences of *VLFY1* and *VLFY2*. Common primers VLFYF0 and VLFYR0 would anneal to sequences in the first and second exons respectively. Paralog specific primers VLFYPSR1 (for *VLFY*1) and VLFYPSR2 (for *VLFY*2) would anneal to sequences in the second exons, and were different by one nucleotide at the 3' end (Fig. 3.3A). The genomic DNA of *V. pubescens* was used for the PCR with different primer pairs. Because the first intron of *VLFY1* and *VLFY2* are different in length (711bp and 346bp respectively), the common primer pair VLFYF0 and VLFYR0 was expected to generate two PCR products from the genomic DNA: a 1112bp fragment from *VLFY1* and a 747bp fragment from *VLFY2*. With VLFYF0 and VLFYPSR1, a PCR product of 1087bp was expected, and the primer pair VLFYF0 and VLFYPSR2 was expected to produce a 722bp PCR product. The PCR with the different primer pairs worked as what was expected

(Fig. 3.3B), so the paralog specific primers could be used to differentiate *VLFY1* and *VLFY2*.

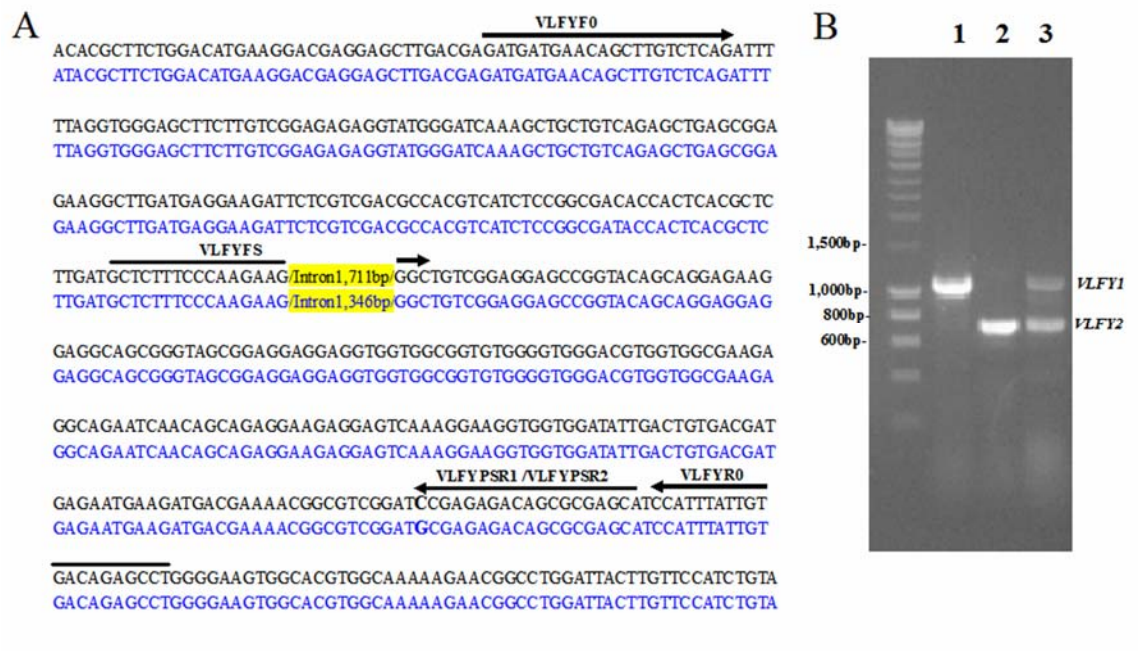

Figure 3.3. Differentiation of *VLFY1* and *VLFY2* by paralog specific primers. (A) The design of the primers. The arrows indicate the location and direction of the primers. The paralogs specific primers VLFYPSR1 and VLFYPSR2 are different by one nucleotide at the 3'end, and the different nucleotide ("G" for VLFYPSR1 and "C" for VLFYPSR2) are shown in bold letters. The sequences of the primers can also be found in Table 3.1. (B) The paralog specific primers could differentiate *VLFY1* and *VLFY2*. Genomic DNA from an individual plant was used in the PCR with the common primer VLFYF0 and one of the primers from the opposite direction: VLFYPSR1 (Lane1), VLFYPSR2 (Lane 2) and VLFYR0 (Lane3).

Another common primer VLFYFS, which spanned the first intron of both genes, was designed to work together with VLFYPSR1 and VLFYPSR2 to detect the expression of *VLFY1* and *VLFY2* in *V. pubescens* by reverse transcription-PCR (RT-PCR). The result indicated that the two genes had expression in both flower types (Fig. 3.4). They were also expressed in the stems, but not the leaves of *V. pubescens* (Fig. 3.4).

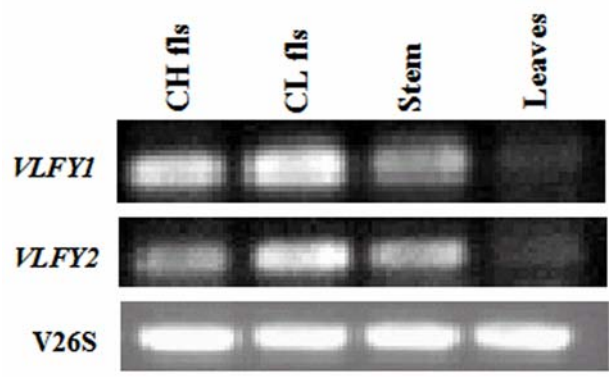

Figure 3.4. Expression of *VLFY1* and *VLFY2* in *V. pubescens*. Transcript of *VLFY1* was amplified with the primer pair VLFYFS and VLFYPSR1, and transcript of *VLFY2* was amplified with the primer pair VLFYFS and VLFYPSR2. *V26S* was used as a control gene. CH fls = chasmogamous flowers; CL fls = cleistogamous flowers.

The expression of *VLFY1* and *VLFY2* was then studied throughout the development of both chasmogamous and cleistogamous flowers, to test whether there were temporal expression differences between the two genes. Both chasmogamous and cleistogamous flowers (from tiny visible buds to mature flowers) of *V. pubescens* were collected from the field. The flowers were divided into different developmental stages based on the length of their sepals, which was considered an index to evaluate the age of flowers (Mayers and Lord, 1983b). In this way, the development of the chasmogamous flowers

was divided into six stages, and the development of the cleistogamous flowers was divided into four stages (Fig. 3.5A). Semi-quantitative RT-PCR indicated that for both chasmogamous and cleistogamous flowers, the expression of *VLFY1* and *VLFY2* was greater when the flowers were younger (e.g. stage 1 and 2) (Fig. 3.5B). Their expression declined gradually as the flowers got older, and almost no expression was detected in mature flowers (Fig. 3.5B).

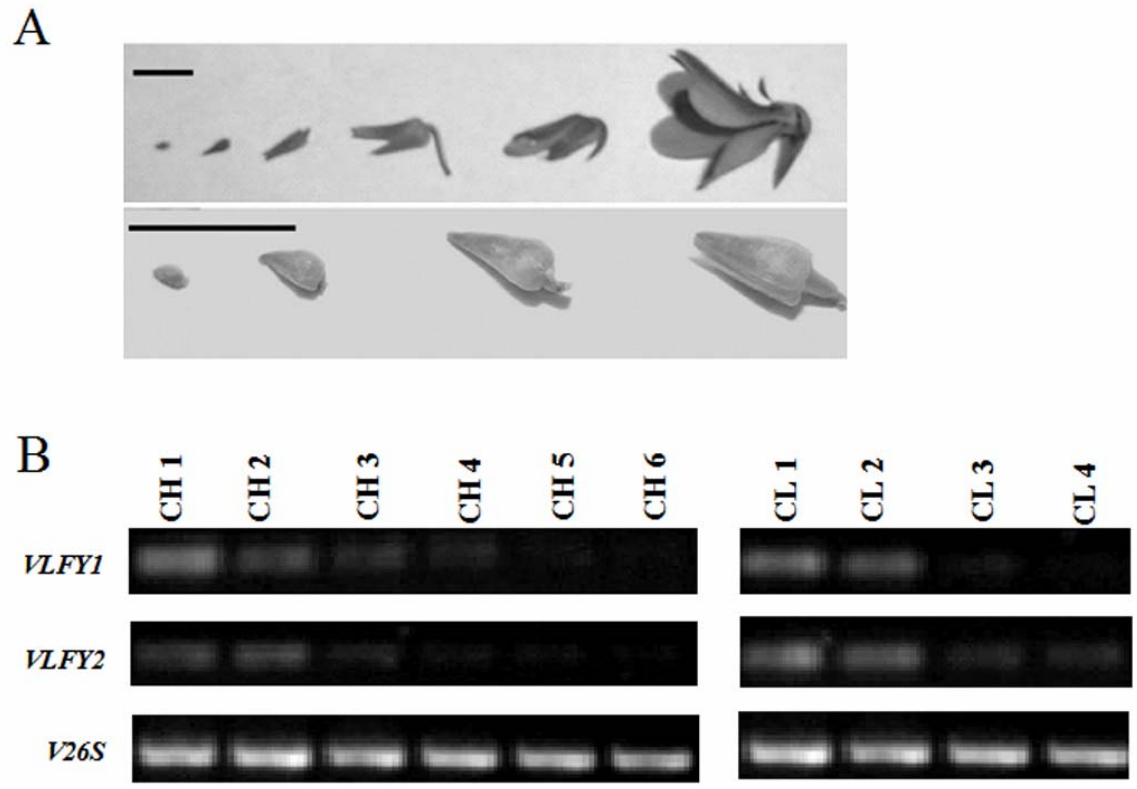

Figure 3.5. Expression of *VLFY1* and *VLFY2* throughout the development of chasmogamous flowers and cleistogamous flowers of *V. pubescens*. (A) Chasmogamous and cleistogamous flowers at different developmental stages. The developmental stages were divided based on the length of the sepals. The top panel shows chasmogamous flowers at stages 1 to 6 (from the left to the right), and the bottom panel shows cleistogamous flowers at stages 1 to 4(from the left to the right). The scale bar in each panel equals to 0.5cm. (B) Gel image of semi-quantitative RT-PCR showing the expression of *VLFY1* and *VLFY2* at different flower developmental stages. Transcript of VLFY1 was amplified with the primer pair VLFYFS and VLFYPSR1, and transcript of VLFY2 was amplified with the primer pair VLFYFS and VLFYPSR2. The flower type (CH fls = chasmogamous flowers; CL fls = cleistogamous flowers) and developmental stages (e.g. 1, 2) are indicated on the top of the image. The 26S rRNA of *V. pubescens* (*V26S*) was used as the control and is shown at the bottom.

Discussion

Viola pubescens *has at least two* LFY *orthologs*

Two *LFY* orthologs, *VLFY1* and *VLFY2*, were cloned from *V. pubescens*. Furthermore, the genomic sequences of both genes had no recognition site for either *EcoR*V or *Hind*III, but three bands were detected by Southern Blot when the genomic DNA was cut with them (Fig.3.1B). Thus there is at least a third *LFY* ortholog present in *V. pubescens* that remains to be identified. Duplication of the *LFY* ortholog was previously thought a rare event in angiosperms (Cronk, 2001); however, more and more plants possessing two or more *LFY* orthologs (e.g. tobacco, eucalypt, apple, corn, and some Lamialean plants) were reported. These duplicate copies are usually considered results of whole genome duplication (polyploidydization) events (Kelly et al. 1995; Southerton et al., 1998; Wada et al., 2002; Bomblies et al., 2003; Aagaard et al., 2005). *Viola pubescens* is a purportedly diploid plant with n=6 chromosomes, in a Northern Hemisphere sublineage of the genus characterized by a base chromosome number of n=6. However, more primitive Latin American lineages that are ancestral to most Northern Hemisphere groups possess high chromosome numbers (e.g., n=27 or n=30, Ballard et al. 1996). Thus, one explanation for the multiple *LFY* orthologs is that they represent duplicative loci stemming from an ancient polyploid ancestor of *Viola*, which subsequently underwent diploidization but retained different loci. Another explanation is that the source of these paralogs could be gene duplication rather than whole genome duplication. A third, less likely, explanation is a hybrid origin of *V. pubescens* or at least

of the specimens used for these studies, although *V. pubescens* is not currently known to hybridize with other *Viola* species.

VLFY1 ***and*** **VLFY2** ***function together in both chasmogamous and cleistogamous flowers***

Paralog specific primers were used in the RT-PCR to differentiate the expression between *VLFY1* and *VLFY2*. The results indicated that both genes were expressed in flowers, and the expression of neither of them was limited a specific floral type (Fig.3.4). In addition, both of them had increased expression in young floral buds but quickly decreased thereafter (Fig.3.5B). The expression pattern of *VLFY1* and *VLFY2* closely resembled that of *LFY*, *FLO* and *AFL1* (*FLO*: the *LFY* ortholog in *Antirrhinum*; AFL: the *LFY* ortholog in apple), whose peak expression was observed in newly-initiated floral primordia (Coen et al., 1990; Blazquez et al., 1997). Thus, *VLFY1,2* appeared to be functional *LFY* orthologs in *V. pubescens* with an evolutionary conserved function in floral induction.

The expression patterns of *LFY* orthologs in vegetative tissues are quite different among species. Some plants (e.g. tobacco, pea, tomato, and poplar) exhibit *LFY* expression in leaves (Kelly et al, 1995; Hofer et al., 1997; Molinero-Rosales, 1999; Rottmann et al., 2000), while no expression of *LFY* orthologs was detected in *Antirrhinum*, apple and grapevine cv. Riesling (Coen et al., 1990; Wada et al., 2002; Joly et al., 2004). In *V. pubescens*, *VLFY1* and *VLFY2* were also not expressed in leaves. *LFY* is expressed in inflorescence (floral stem) of Arabidopsis (https://iii.genevestigator.ethz.ch/at/index.php). Similarly, *VLFY1* and *VLFY2* are

expressed in stem of *V. pubescens*, although the two *LFY* orthologs *AFL1* and *AFL2* do not have expression in stem (Wada et al., 2002).

In *V. pubescens*, no significant difference was detected in the expression of *VLFY*1 and *VLFY2* in different organs or at different flower developmental stages (Fig.3. 4 and Fig.3.5A). On the contrary, different *LFY* orthologs in other species were reported to be functionally unequal. For example, *Pinus radiata* has two *LFY* orthologs: *NEEDLY* (*NLY*) and *PRFLL*. *NLY* was expressed during vegetative development, while the transcript of *PRFLL* was detected in developing male cones (Mouradov et al., 1998; Melleriowicz et al., 1998). *AFL1* and *AFL2* are two *LFY* orthologs in apple. Wade et al. (2002) only detected *AFL1* in the floral shoot apex, but expression of *AFL2* was also observed in the vegetative apex, floral organs, and roots. The similarities between VLFY1 and VLFY2 were higher than any known pairs of LFY-like proteins in a single species (e.g. tobacco, apple, corn), this fact might be one of the reasons why *VLFY1* and *VLFY2* did not appear to have divergent roles. However, promoter analysis revealed different regulatory elements in *VLFY1* and *VLFY2*. The tentative explanation is that the two genes detect different environmental signals, and the activation of one soon upregulates the other one, then they function together in the flower development.

In summary, two *LFY* orthologs (*VLFY1* and *VLFY2*) were cloned from *V. pubescens*, a violet with the chasmogamous-cleistogamous mixed breeding system, and evidence supports the existence of a third as yet unidentified ortholog. The two characterized genes encode proteins with more than 98% similarities, but their promoters harbored different *cis*-regulatory sequences. The two genes function together in both

chasmogamous and cleistogamous flowers, with an increased expression in young floral buds. Further research needs to be done to investigate the specific regulation and roles of each gene in detail.

Chapter 4: DIFFERENTIAL EXPRESSION OF THE ABC FLORAL GENE ORTHOLOGS PLAYS A ROLE IN THE MIXED BREEDING SYSTEM OF *VIOLA PUBESCENS* (VIOLACEAE)

Introduction

Plants that can produce both open (chasmogamous) and closed (cleistogamous) flowers on the same individual occur in more than 50 angiosperm families (Lord, 1981; Culley and Klooster, 2007). The open and showy chasmogamous flowers usually do out-crossing to produce seeds, while the closed and underdeveloped cleistogamous flowers have to produce seeds by self-pollination. The mixed chasmogamous-cleistogamous breeding system increased seed production and represents an evolutionarily successful reproductive strategy (Uphof, 1938; Lord, 1981; Masuda et al., 2001; Lu, 2002). Scientists have been interested in plants with the mixed breeding system for a long time, and a large body of morphological, ecological and population biological work has been conducted (Beattie, 1971, 1974; Mayers and Lord, 1983a, 1983b; Masuda and Yahara, 1992; Culley, 2002). But the molecular basis underlying the floral dimorphism remains largely unknown. Studying the molecular mechanisms of chasmogamous and cleistogamous flowers can advance our knowledge of the evolution and maintenance of the mixed breeding system.

As compared to chasmogamous flowers, cleistogamous flowers exhibit various degrees of reductions and modifications in different floral parts, and precocious sexual maturation is another distinct character associated with cleistogamous flowers (Uphof, 1938; Lord, 1981; Campbell et al., 1983). For example, in *Collomia grandiflora* Dougl. ex Lindl., smaller cell numbers and sizes in cleistogamous petals resulted in a reduced cleistogamous corolla (Minter and Lord, 1983b). On the other hand, pollen meiosis

occurred significantly earlier in the cleistogamous flowers than in the chasmogamous flowers (Minter and Lord, 1983b).

The ABC model of flower development states that three classes (A, B, and C) of homeotic transcription factors are essential for the initiation of different floral organs (Coen and Meyerowitz, 1991; Theissen, 2001). Specifically, class A genes specify sepals and work together with class B genes to control petal development. Class B and C collaborate to give rise to stamens while carpels are determined by C class genes (Coen and Meyerowitz, 1991; Theissen, 2001). Additional research extended the ABC model to the ABCDE model, with D class genes specifying ovules and E class genes contributing to the determination of all four floral organs (Theissen and Saedler, 2001; Jack, 2004; Krizek and Fletcher, 2005). In *Arabidopsis thaliana* (L.) Heynh., the A class genes are *APETALA1* (*AP1*) and *APELATA2* (*AP2*); the B class consists of *APETALA3* (*AP3*) and *PISTILLATA* (*PI*); *AGAMOUS* (*AG*) is the only known member of the C class; and *SEPALLATA1, 2, 3, 4* (*SEP1, SEP2, SEP3, SEP4*) belong to the E class (Theissen and Saedler, 2001; Jack, 2004; Krizek and Fletcher, 2005). Jack (2004) suggested *SEEDSTICK*, *SHATTERPROOF1* and *2* be considered as D class genes in *Arabidopsis* based on their functions in ovule development.

Besides their organ-identity functions, ABCDE floral genes activate different downstream effectors to control the elaborate morphogenesis and growth programs of different floral organs (Jack, 2004; Krizek and Flecher, 2005). Quite a few such effectors were identified in recent years (Sablowski and Meyerowitz, 1998; Ito, et al. 2004; Takeda, et al, 2004; Lee et al., 2005; Szecsi, et al, 2006). The expression of *RABBIT*

EARS (*RBE*), which encodes a SUPERMAN-like zinc finger protein, is downstream of *AP1*. *RBE* affects the organ shape on the second whorl, regardless of the organ identity (Takeda et al, 2004). *NAC-LIKE, ACTIVATED BY AP3/PI* is directly induced by the *AP3/PI* heterodimer and is functional in the transition between cell division and cell expansion in developing stamens and petals (Sablowski and Meyerowitz, 1998). *AG* exerts its function on microsporogenesis by activating different downstream regulators such as the *SPOROCYTELESS* (*SPL*) gene (Ito, et al. 2004). *AP3, PI, AG* and *SEP* were also suggested to be positive regulators of the *CRABS CLAW* gene, which is required for nectary and capel development (Lee, et al. 2005). Very recently, *BIGPETALp* (*BPEp*) was discovered to control petal size by interfering with cell expansion. *AP1*, *AP3/PI*, and *SEP* positively regulate *BPEp* while *AG* has a negative influence on its expression (Szecsi, et al, 2006). With more and more labs dedicating to identifying downstream targets of the ABCDE floral genes, the list will continue to be extended.

Considering the facts that chasmogamous and cleistogamous flowers have floral organs adopting different morphologies and growth rates and that the ABCDE floral genes are master regulators of floral organ development, the hypothesis was that differential expression of the ABCDE floral genes plays a role in the production of the two flower types. The present research focused on the ABCE floral gene orthologs in a cleistogamous violet, *Viola pubescens* Aiton (commonly known as yellow-stemmed violet), to test our hypothesis. *V. pubescens* belongs to a genus famous for the large number of species with the chasmogamous-cleistogamous mixed breeding system (Lord, 1981; Culley and Klooster, 2007). This species was chosen for investigation because it is

easily available, produces an abundance of chasmogamous and cleistogamous flowers over the growing season, and is putatively diploid with n=6 chromosomes (Ballard, 1996). *V. pubescens* is a perennial herb, commonly found in the understory of mesic forests in eastern North America. The plant produces chasmogamous flowers in the early spring (mid-April to May). After the forest canopy has fully leafed out, cleistogamous flowers are produced (May to September) (Culley and Wolfe, 2001; Culley, 2002). The ABCE floral gene orthologs of *V. pubescens* were identified by RT-PCR from the floral RNA of *V. pubescens*. Semi-quantitative RT-PCR was conducted to compare the expression of these floral genes in the two flower types, in an effort to understand the roles of the ABCE floral genes in the mixed breeding system.

Materials and Methods

Plant materials

Chasmogamous and cleistogamous flowers of *V. pubescens* at different developmental stages (from visible buds to mature flowers), as well as leaves and stems, were collected from the Ridges Land Laboratory of Ohio University. Plant materials were preserved in RNAlater RNA Stabilizing Reagent (Qiagen, Valencia, California, USA) immediately after collection.

Morphology

Some RNA-later preserved flowers were spared for morphological study. Chasmogamous flowers at full anthesis and cleistogamous flowers right before seed-set were dissected under a binocular microscope by sequentially peeling away whorls of

floral organs to expose the inner ones. Images of each set of floral organs were captured using a digital camera ((Nikon, Japan) attached to the microscope. Sizes of flowers and floral organs were compared by Image J software (http://rsb.info.nih.gov/ij/).

RT-PCR

Total RNA was extracted from flowers, leaves and stems with the RNeasy Plant Mini Kit (Qiagen, Valencia, California, USA) and treated with DNase (Qiagen, Valencia, California, USA). RT-PCR was conducted using the OneStep RT-PCR Kit (Qiagen, Valencia, California, USA). Degenerate primers for the RT-PCRs were designed from the conserved regions of the known *ABC* orthologs in other plant species obtained from GenBank. Sequences of the designed primers are listed in Table 4.1. The RT-PCR program included a reverse transcription step at 50ºC for 30min followed by a step to inactivate the reverse transcriptase at 94ºC for 15min, and then 35 cycles of 30s at 94ºC for denaturation, 1 min for annealing (the annealing temperature varied, depending on the primer pairs, and can be found in Table 4.1.) and 1min at 72ºC for extension. Negative controls without reverse transcriptase were conducted to eliminate the possibility of genomic DNA contamination. RT-PCR products were cloned into the TOPO TA cloning vector (Invitrogen, Carlsbad, California, USA) and then sequenced on an ABI 310 genetic analyzer. The sequences were analyzed with the Basic Local Alignment Search Tool (BLAST) provided by NCBI to confirm their identities.

Semi-quatitative RT-PCR

The *26S rRNA* gene of *V. pubescens* was used as a control gene, and the primer sequences can be found in Table 4.1. Products of the semi-quantitative RT-PCR were analyzed on 1.5% agarose gels by electrophoresis. Relative abundance of each floral gene mRNA was normalized to that of the control gene *V26S* using Image J software (http://rsb.info.nih.gov/ij/).

Table 4.1.

Primer Sequences and Annealing Temperatures for the Amplification of ABCE Floral Gene Orthologs and the 26S rRNA Gene in* Viola pubescens

Primer name	***Primer sequence***	***Annealing Tm (°C)***
VAP1-F2	5’ GGTTGCYKTKATTRTCTTCTC 3’	50
VAP1-R1	5’ GARTCNARATCYTCYCCCA 3’	
VAP2-F2	5’ GTTTAYYTAGGTGGATTTGACAC 3’	60
VAP2-R1	5’ TTATCRTAAGCYCTRGCAGCTTC 3’	
VAP3-F1	5’ CTYACYGTTCTYTGTGATGC 3’	50
VAP3-R1	5’ TACYAAHCCAHMVTGTGGATC 3’	
VPI-F3	5’GAGATAAAGAGGATAGAGAAC 3’	50
VPI-R1	5’ CACAACACAYCCATATARATA 3’	
VAG-F1	5’ GGMAARATWGARATMAAGAGGA 3’	58
VAG-R1	5’ TTYTCMAVYTGYTTBAGYTCC 3’	
VSEP3-F2	5’ GARGTWGCTCTSATCATCTTCTC 3’	60
VSEP3-R2	5’ CNAGATCYTCBCCVADVAGRTTCC 3’	
V26S-F	5’ GCACCTGAGTCCGACGTC 3’	58
V26S-R	5’ GCGGCGAAGCAGCCAAGG 3’	

* Degenerate nucleotide codes are used, with W=A or T; S=C or G; R=A or G; Y=C or T; K=G or T; M=A or C; B=C, G or T; D=A, G or T; H=A, C or T; V=A, C or G; N=A, C, G, or T.

Results

Morphological differences between the two flower types

Both types of flowers had five well-developed sepals, although the sepals of cleistogamous flowers were smaller (Fig. 4.1A,B). Chasmogamous flowers had five large and showy petals with the lowest one protruding slightly at the base into a "spur", characteristic of *Viola* flowers (Fig 4.1C). Five stamens of chasmogamous flowers formed a cone surrounding the pistil, and the lowest two stamens had noticeable nectar glands attached to them (Fig. 4.1D). In contrast, the petals and stamens in cleistogamous flowers were quite rudimentary in appearance. The "spur" on the lowest petal was scarcely evident, and no nectar glands were observed on the stamens (Fig. 4.1E). Some petals were reduced to filamentous structures, and some stamens had no pollen sacs (pictures not shown). Although the overall size of cleistogamous flowers was about 1/3 that of chasmogamous flowers (Fig. 4.1A,B), the ovary of cleistogamous flowers was only slightly smaller than those of chasmogamous flowers (Fig. 4.1F,G). Thus ovaries develop to a further extent in cleistogamous flowers relative to in chasmogamous flowers. The tightly curved style of cleistogamous flowers was reported to facilitate self-pollination (Beattie, 1971) (Fig. 4.1G).

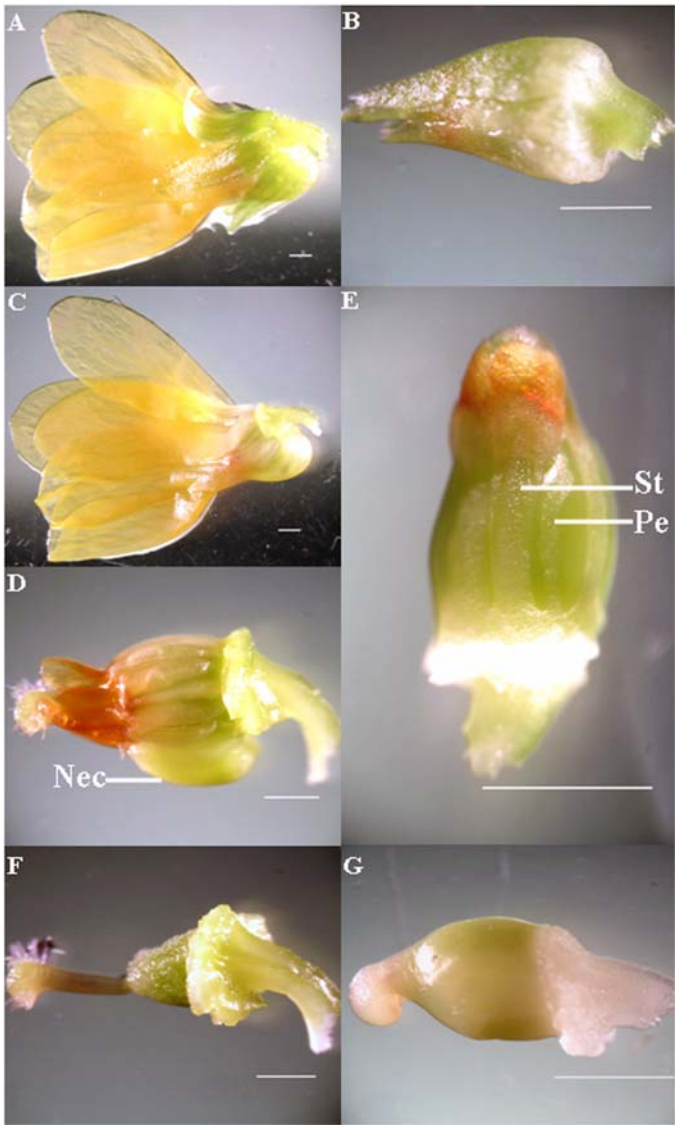

Figure 4.1. Dissection of chasmogamous and cleistogamous flowers of *Viola pubescens*. Chasmogamous flowers at full anthesis and chasmogamous flowers right before seed-setting were dissected to show their floral organs. A, C, D, F: chasmogamous flowers. B, E, G: cleistogamous flowers. (A) An intact chasmogamous flower. (B) An intact cleistogamous flower. (C) A chasmogamous flower without sepals. (D) A chasmogamous flower without sepals and petals (Nec: nectary gland). (E) A cleistogamous flower without sepals (Pe: petal; St: stamen). (F) Pistil of a chasmogamous flower. (G) Pistil of a cleistogamous flower. The scale equals to 1mm.

Identification of the ABCE floral gene orthologs

RT-PCR products about 300-500bp in length were obtained from flower RNAs with specific degenerate primer pairs (Fig. 4.2). The identities of the RT-PCR products were verified by sequencing and BLAST analysis. The genes generating RT-PCR products that share high identities (>80%) with the ABCE floral genes in other plants were considered ABCE floral gene orthologs in *V. pubescens*. They were tentatively named *VAP1*, *VAP2, VAP3, VPI*, *VAG* and *VSEP3* respectively. The control gene, *26S rRNA* in *V. pubescens*, was identified in a similar way and designated *V26S.* The sequences of *VAP1*, *VAP2, VAP3, VPI*, *VAG* and *VSEP3* were submitted to Genbank and could be found in under the accession numbers: EF442781, EF453370, EF453371, EF564798, EF453372, and EU169457.

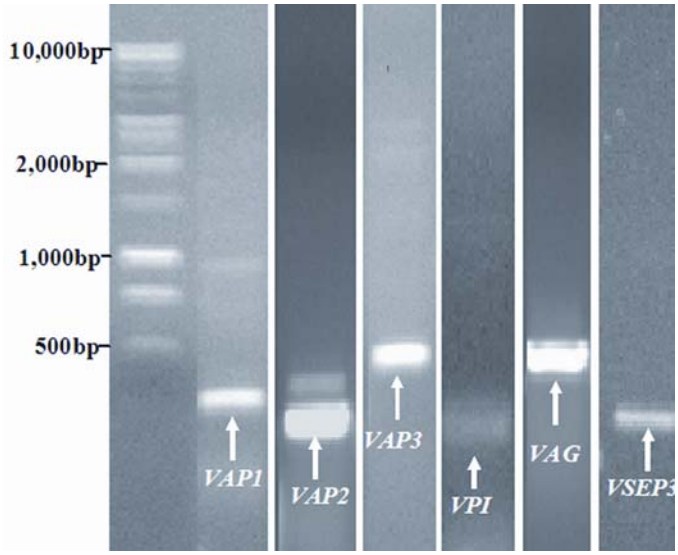

Figure. 4.2. Identification of the ABCE floral gene orthologs of *V. pubescens*. The bands at the arrows were RT-PCR products amplified from floral RNA of *V. pubescens* with specific degenerate primers. The sequences of the RT-PCR products showed high similarities (>80%) to the known ABCE floral genes in other plants. The genes generating these RT-PCR products were named *VAP1*, *VAP2*, *VAP3*, *VPI*, *VAG* and *VSEP3* respectively. The left most lane is the molecular marker, and the sizes of some bands are indicated.

***Expression of* VAP1, VAP2, VAP3, VPI, VAG *and* VSEP3 *in* V. pubescens**

The expression of *VAP1*, *VAP2*, *VAP3*, *VAPI*, *VAG* and *VSEP3* were studied in flowers, leaves and stems of *V. pubescens* by semi-quantitative RT-PCR, with the *26S rRNA* gene of *V. pubescens* used as a loading control (Fig. 4.3). All genes exhibited expression in both chasmogamous and cleistogamous flowers (Fig. 4.3). Their expression levels in leaves and stems varied. *VAP1* was expressed in stems but was barely detectable in leaves (Fig. 4.3). The RNA of *VAP2* was observed in both leaves and stems, although to a lesser degree than in flowers (Fig. 4.3A). For the B, C and E classes of genes, little expression was detected in leaves and stems (Fig. 4.3).

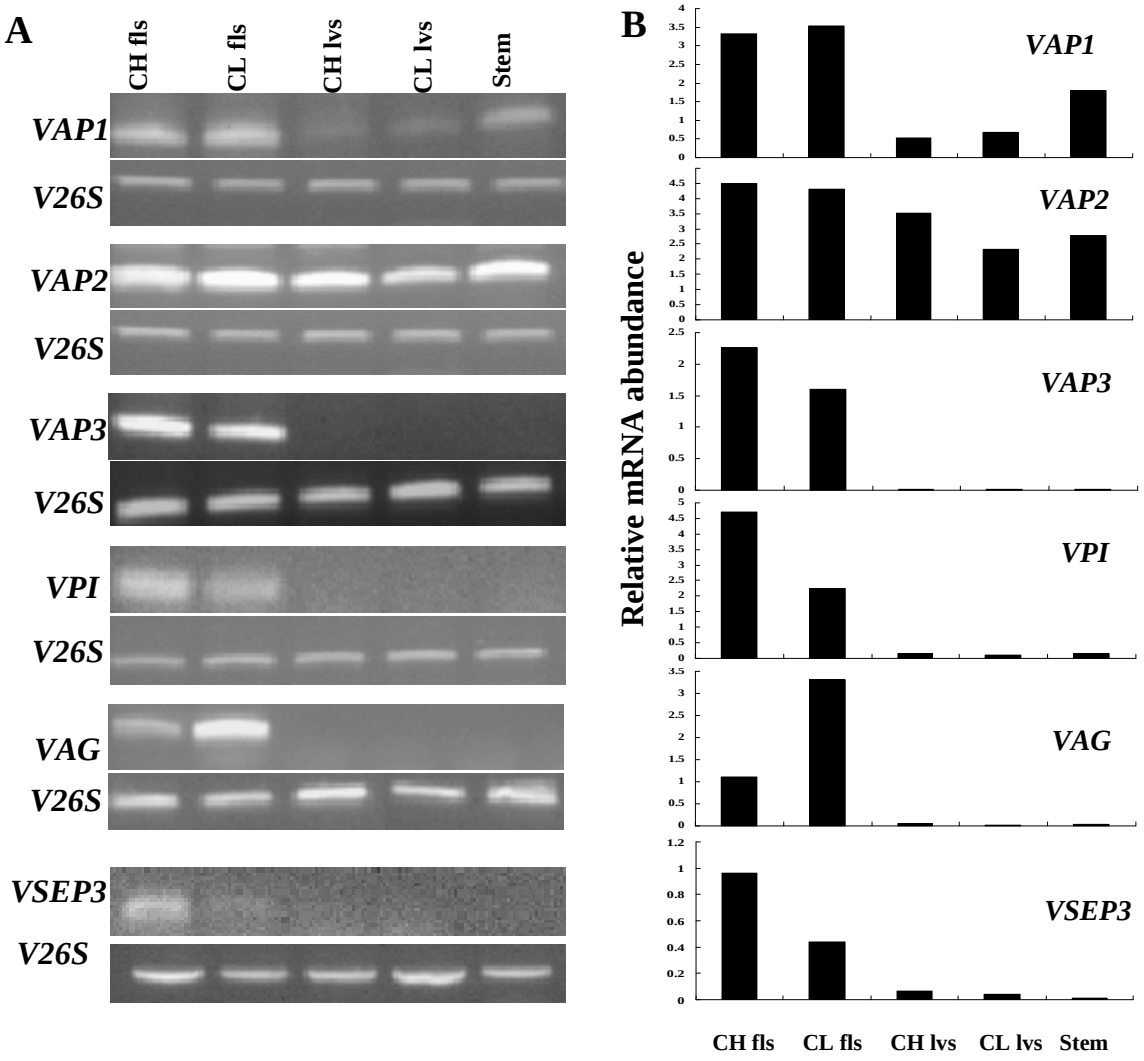

Figure 4.3 Expression of the ABCE floral gene orthologs in *V. pubescens.* Expression of the ABCE floral gene orthologs were compared in chasmogamous flowers (CH fls), cleistogamous flowers (CL fls), chasmogamous leaves (CH lvs, leaves subtending chasmogamous flowers), cleistogamous leaves (CL lvs, leaves subtending cleistogamous flowers) and stems (Stem) by semi-quantitative RT-PCR with specific degenerate primers. The *26S rRNA* gene of *V. pubescens* (*V26S*) was used as the control gene. A. Gel image of semi-quantitative RT-PCR showing the expression of *VAP1*, *VAP2*, *VAP3*, *VPI*, *VAG* and *VSEP3* in *V. pubescens*. B. Relative abundances of the mRNAs of *VAP1*, *VAP2*, *VAP3*, *VPI*, *VAG* and *VSEP3* to that of V26Sin *V. pubescens*.

Expression of VAP1, VAP2, VAP3, VPI, VAG *and* VSEP3 *in chasmogamous and cleistogamous flowers*

Comparison of the expression of the ABCE floral gene orthologs of *V. pubescens* in its chasmogamous and cleistogamous flowers was conducted. Different expression patterns were observed, although the expression profiles of genes in the same class were quite similar. The expression patterns of the A class genes *VAP1* and *VAP2* were similar in the two types of flowers (Fig. 4.3). The expressions of B class genes *VAP3* and *VPI* were more pronounced in chasmogamous than cleistogamous flowers (Fig. 4.3). The C class gene *VAG* was expressed to a much weaker degree (about three times less) in chasmogamous flowers as compared to in cleistogamous flowers (Fig. 4.3). For the E class gene *VSEP3*, its expression level was higher in chasmogamous flowers than in cleistogamous flowers (Fig. 4.3)

Discussion

VAP1, VAP2, VAP3, VPI, VAG *and* VSEP3 *are ABCE floral gene orthologs in* V. pubescens

The ABCDE model of flower development, derived initially from studies of two eudicot model plants *Arabidopsis thaliana* and *Antirrhinum majus L.*, was developed to explain how four whorls of floral organs are controlled by three classes of homeotic genes (Coen and Meyerowitz, 1991; Theissen, 2001). The simplicity of the ABCDE model promoted the identification and characterization of ABCDE orthologs in many other plant species, and conserved roles of the ABCDE orthologs were confirmed across

angiosperms and gymnosperms (Whipple et al., 2004; Zhang et al. 2004; Krizk and Fletcher, 2005; Zahn et al., 2005).

We have identified two A class genes (*VAP1* and *VAP2*), two B class genes (*VAP3*, *VPI*), one C class gene (*VAG*), and one E class gene (*VSEP3*) by RT-PCR from *V. pubescens*, a violet species with a chasmogamous-cleistogamous mixed breeding system. Sequence analysis indicated high levels of identity between them and the known ABCE floral gene orthologs in other plants. Their expression in both chasmogamous and cleistogamous flowers (both types of flowers have sepals, petals, stamens and carpels) demonstrated their roles in flower development in *V. pubescens* (Fig. 4.3). In addition, the expression patterns of *VAP1*, *VAP2*, *VAP3*, *VPI*, *VAG* and *VSEP3* in different plant organs (flowers, leaves and stems) were similar to those of their counterparts in *Arabidopsis* (Fig. 4.3). Taken together, *VAP1*, *VAP2*, *VAP3*, *VPI*, *VAG* and *VSEP3* are ABCE floral gene orthologs in *V. pubescens* with evolutionarily conserved functions, just as these gene systems have been documented in other species.

***Differential expression patterns of the ABCE floral gene ortholog contribute to the mixed breeding system in* V. pubescens**

Generally, cleistogamous flowers have all four whorls of floral organs, but these are substantially reduced or even rudimentary in some instances. Accelerated sexual organ maturation is also typical in cleistogamous flowers (Uphof, 1938; Lord, 1981; Campbell et al., 1983), such that perianth parts are arrested in development at a much earlier chronological stage when the androecium and gynoecium mature to become functional.

According to the ABCDE model, sepals are controlled by the A class genes (Coen and Meyerowitz, 1991; Theissen and Saedler, 2001). The two A class genes in *V. pubescens*, *VAP1* and *VAP2*, exhibited similar expression in both types of flowers (Fig. 4.3). In *V. pubescens*, sepals of both types of flowers were well developed, surrounding and protecting the inner floral organs (Fig. 4.1A, B). The equivalent expression of *VAP1* and *VAP2* was thought to ensure the development of sepals in the two flower types. B class genes function in development of the petals and stamens (Coen and Meyerowitz, 1991; Theissen and Saedler, 2001). Underdevelopment of petals and stamens was apparent in the cleistogamous flowers of *V. pubescens* (Fig. 4.1C,D,E), as is typical of many other plants with the chasmogamous-cleistogamous mixed breeding system (Uphof, 1938; Lord, 1981; Campbell et al, 1983). Corresponding to the rudimentary state of petals and stamens, distinctly lower expression of *VAP3* and *VPI* was detected in cleistogamous flowers of *V. pubescens* (Fig.4.3). Because the E class genes are involved in the development of all floral organs, the reduced expression of *VSEP3* in the morphologically rudimentary cleistogamous flowers is also not surprising (Fig. 4.3).

Expression of the C class gene *VAG* was dramatically greater in cleistogamous flowers than in chasmogamous flowers (Fig. 4.3). In *Arabidopsis*, *AG* specifies reproductive floral organs and later plays essential roles in their development and maturation (Bowman et al. 1991; Ito et al., 2004; Skinner et al. 2004; Gomez-mena et al., 2005). For example, *SPL*, a gene controlling microgenesis, is upregulated by *AG* (Ito et al., 2004). High expression of *AG* in ovule primordia and integuments indicates the contribution of *AG* to the development of ovules (Bowman et al. 1991; Skinner et al.

2004). Maturation of reproductive organs is much earlier and faster in cleistogamous flowers than in chasmogamous flowers (Uphof 1938; Lord, 1981; Campbell et al., 1983). In *V. ordorata*, pollen meiosis occurs about eight days later in chasmogamous flowers than in cleistogamous flowers, and it takes 15 more days for chasmogamous flowers to mature (Mayers and Lord, 1983a, 1983b, 1984). At the same time, chasmogamous flowers of *V. odorata* L. are about four times bigger than cleistogamous flowers, but their ovaries are only 1.25 times bigger than those of cleistogamous flowers (Mayers and Lord, 1983b). Similarly, in *V. pubescens*, cleistogamous flowers have a size about 1/3 that of chasmogamous flowers (Fig. 4.1A, B), but their ovaries were only slightly smaller than those of chasmogamous flowers (Fig.4.1F, G). Thus, in both violet species, ovaries actually occupy a larger proportion of cleistogamous flowers than ovaries of chasmogamous flowers. Despite all the differences between the two flower types, their seeds exhibit no significant differences in number per capsule, mean seed mass or seedling performance in *V. pubescens* (Culley, 2002). Taken together, the increased expression of *VAG* in the cleistogamous flowers of *V. pubescens* was thought to accounts for their faster maturation and competent ability to produce seeds as the big chasmogamous flowers.

ABCE floral genes in plants with different forms of flowers

Many plants produce more than one type of flower to achieve special reproductive advantage. Homeotic floral genes, especially those in the B and C classes, have been studied in several dioecious and monoecious species to gain insights into the molecular basis of unisexual flowers. Different spatial and temporal performances of floral

homeotic genes in male and female flowers were observed, suggesting important roles in the floral-sex determination in these plants (Hardennack et al., 1994; Ainsworth et al., 1995; Ambrose et al. 2000; Kater et al. 2001; Park et al., 2003; Di Stillo et al., 2005; Pfent, et al., 2005). The terminal inflorescence of sunflower (*Helianthus annuus* L.) is composed of two flower forms: peripheral ray (sterile) flowers and central disc (fertile) flowers, and the B and C classes of floral genes were expressed at different levels in the two types of flowers (Dezar, et al., 2003). This study also detected differential expression of B, C and E classes of genes in chasmogamous and cleistogamous flowers of *V. pubescens*.

Overall, the data in these studies indicate the involvement of the ABCE floral homeotic genes in the production of different flowers by the same species. These results require further investigation into the details of the molecular regulation of the floral homeotic genes in these and other plants with unusual breeding systems. In most cases of the chasmogamous-cleistogamous mixed breeding system, the two types of flowers do not occur simultaneously. Different environmental conditions, including photoperiod, light availability, temperature, and nutritional conditions, as well as different plant growth hormone levels, were proposed to influence the production of specific flower types (Uphof, 1938; Lord, 1981; Campbell et al., 1983). Thus, the ABC(D)E floral genes of cleistogamous flowers might be subjected to the regulation of exogenous environmental and endogenous hormonal conditions, resulting in different expression patterns and eventually the production of two types of flowers. Studying the regulation of the ABC(D)E floral gene orthologs, especially with regard to potential hormone

gradients and environmental signaling cues, will provide more insight into the basis of the chasmogamous-cleistogamous mixed breeding system.

In summary, we have identified the ABCE floral gene orthologs in a cleistogamous violet *V. pubescens* and compared its expression in both chasmogamous and cleistogamous flowers. The results indicated that these floral genes function in the two flower types. The different expression patterns in chasmogamous and cleistogamous flowers, especially the heightened expression of the C class gene in cleistogamous flowers, suggested contributions of the ABCE floral genes in the differentiation of the two types of flowers in violets. Future studies will probe the details of floral homeotic gene regulation as part of a broader research program on the evolution of the chasmogamous-cleistogamous mixed breeding system and its underlying molecular basis.

Chapter 5. CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

This research project focused on flower development in *Viola pubescens* Aiton from a molecular point of view, in an effort to gain an understanding of the molecular mechanisms involved in the chasmogamous-cleistogamous mixed breeding system. *Viola pubescens* is a widespread North American species in the genus *Viola*, which has far more species with both chasmogamous and cleistogamous flowers than any other genus. Chasmogamous flowers of *V. pubescens* are produced in early spring, when the canopy is open and cleistogamous flowers are produced in summer, when the canopy is closed.

This study focused on genes participating in the gibberellic acid (GA) induced flowering pathway, in which GA, floral meristem identity gene *LFY* and the ABCE floral organ identity genes work in turn to give rise to flowers. The GA induced flowering pathway was chosen because researchers previously demonstrated that treatment of GA could induce chasmogamous flowers from cleistogamous flowers in a few studied species (Lord, 1980; Raghuvanshi et al., 1981, Minter and Lord, 1983a). The hypothesis of the research is that in *Viola pubescens*, the genes in the GA induced flowering pathways are differentially expressed between the chasmogamous and cleistogamous flowers.

GA

Two genes (*VGA20ox and VGA3ox*), both of which encode key enzymes in the biosynthesis of GA, had increased expression in chasmogamous flowers compared to in cleistogamous flowers of *V. pubescens*. This result is consistent with previous reports

that big and showy chasmogamous flowers require more input of GA (Lord, 1981). The fluctuation in the expression levels of *VGA20ox* and *VGA3ox* were proposed to be influenced by the different light conditions under which the two flower types were produced. More light is available in the early spring when chasmogamous flowers are produced while less light can reach the plants in summer when cleistogamous flowers are produced. On the other hand, unlike in the cases of *Lamium amplexicaule* L., and *Collomia grandiflora* Dougl. ex Lindl., spraying *V. pubescens* with GA3 before the emergence of cleistogamous flowers only resulted in more cleistogamous flowers, but no appearance of chasmogamous flowers. The inability of GA alone to induce chasmogamous flower production in *V. pubescens* was explained by the much earlier divergence in the development of the chasmogamous and cleistogamous flowers in *Viola* than in *L. amplexicaule*, and *C. grandiflora*. Other unknown factors seemed to be more essential than GA in determining the opening status of the flowers.

LFY *orthologs*

The *LFY* orthologs function as switches from vegetative growth to reproductive growth in *Arabidopsis* thaliana (L.) Heynh. and many other plants. *LFY* is usually considered a low-copy gene and duplicated *LFY* genes have been found only in polyploid species such as tobacco and apple (Kelly et al. 1995; Southerton et al., 1998; Wada et al., 2002; Bomblies et al., 2003; Aagaard et al., 2005). Nevertheless, two *LFY* orthologs (*VLFY1* and *VLFY2*) were cloned from the putatively diploid (n=6) plant *V. pubescens*, and there is clear evidence for the presence of a third ortholog. The multiple *LFY*

orthologs could be inherited from an ancient polyploid ancestor prior to diploidization or condensation of many chromosomes into a few pairs, or could result from gene duplication rather than whole genome duplication. The coding regions of *VLFY1* and *VLFY2* share high similarity (98% similarities in the amino acid sequences of the two proteins), but their introns are quite divergent, indicating that the duplication (whatever the scope) is quite ancient. In addition, their promoters had different *cis*-regulatory elements, suggesting differences in their regulation.

A gene expression study detected no significant differences between *VLFY1* and *VLFY2*. Both genes functioned in the development of both chasmogamous and cleistogamous flowers, with more expression in the early stages. Thus, *VLFY1* and *VLFY2* seem to be collaborative in *V. pubescens*. Transgenic *Arabidopsis* overexpressing *VLFY1* and *VLFY2* separately are being developed to assess their individual roles.

ABCE floral organ identity genes

The ABCE classes of floral genes are essential for initiation as well as development of the various floral organs. In *V. pubescens*, both chasmogamous and cleistogamous flowers have four whorls of floral organs: sepals, petals, stamens and carpels. But the floral organs developed to different extents: the sepals of both flower types are well developed; petals and stamens of cleistogamous flowers are quite rudimentary in appearance; the ovaries of cleistogamous flowers are only slightly smaller than those of the chasmogamous flowers, although a whole cleistogamous flower is about 1/3 the size of a chasmogamous one.

The expression of the ABCE floral genes was compared between chasmogamous and cleistogamous flowers of *V. pubescens*. The two A class genes *VAP1* and *VAP2*, which control sepal development, had similar expression levels. The B class genes function in petal and stamen development, and *VAP3* and *VPI* (B class gene identified in *V.pubescens*) had decreased expression in cleistogamous flowers as compared with chasmogamous flowers. Carpels are controlled by C class genes, and the expression of *VAG* (the C class gene of *V. pubescens*) is greater in cleistogamous flowers than in chasmogamous flowers. The differential expression patterns of the ABCE classes of genes in *V. pubescens* are in accordance with the morphological differences between the two flower types and expectations of differential heterochronic expression of the homeotic genes in early development of undifferentiated young flower buds.

In summary, most of the floral genes examined here exhibited differential expression patterns between chasmogamous and cleistogamous flowers, which supported the initial hypothesis of differential and heterochronic regulation. *V. pubescens* is found in the understory of mesic forests. The chasmogamous flowers are produced in the early spring when abundant light can reach the forest floor; while cleistogamous flowers are produced in summer when limited light can penetrate the leafy canopy to reach the plants. Thus, the differential expression patterns are proposed to be responses to the different environmental conditions under which the two types of flowers are produced, and contributed to the production of chasmogamous and cleistogamous flowers. This study presents the first molecular data concerning the genetic basis of the chasmogamous-cleistogamous mixed breeding system and encourages further molecular

genetic research on this fascinating evolutionary phenomenon in violets and other plant groups exhibiting this life history trait.

Future Directions

Examine the floral gene expression prior to floral meristem formation

Expression of many floral genes are actually initiated before the formation of floral meristems, an event occurring much earlier than the flowers are visible to naked eyes (e.g., floral meristems of Arabidopsis require two weeks to develop into visible floral buds). The presented research used flowers after they were big enough to be visible. Because chasmogamous flowers and cleistogamous flowers in *Viola* diverge early in development (Mayers and Lord, 1983a), examination of the floral genes before and around floral meristem formation is an more accurate way to evaluate the initiation time and initial expression levels of the floral genes.

Decipher roles of the multiple **LFY** *orthologs*

The presence of multiple *LFY* orthologs in the diploid *V. pubescens* is quite unusual. What is the relationship among them? Do they each carry specific functions or they work redundantly? Does one of them determine the chasmogamous or cleistogamous fate of the floral meristem? How are they regulated? These questions can not be answered without the complete sequence information and functional assays of each of the *LFY* orthologs.

Study the regulation of the B and C classes of genes

The B and C classes of genes of *V. pubescens* were differentially expressed, with decreased expression of B class genes and increased expression of C class genes in cleistogamous flower as compared to chasmogamous flowers. How these genes are regulated differently in the two flower types is an interesting question. Identification of their *cis*-regulatory elements, trans-factors, and / or epigenetic regulatory mechanisms may provide insights to this question.

Identify other floral genes

Many other genes beyond those on the GA-induced flowering pathway function in flowering. We should also look into them, especially those upstream of the *LFY* orthologs, to get a broader picture of the molecular mechanisms of the chasmogamous-cleistogamous mixed breeding system.

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