MOLECULAR CHARACTERIZATION OF PHOTOPERIODIC FLOWERING IN STRAWBERRY (*Fragaria* SP.)

By

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To my parents,
who always believed in me, and taught me to love knowledge and the natural world.

To the memory of my grandfather, Jackson Lee Stewart, Jr.,
who introduced me to agriculture, and who will forever be my image of the American farmer.

To my children, Zea and Adina,
who have been a constant inspiration and joy to me,
and reminders of the things in life that are truly important.

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in her willingness to sacrifice for my academic and professional pursuits,
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By

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The initiation of flowering is a critical developmental transistion for most plant species, affecting reproductive potential and evolutionary success. The timing of this event in strawberry (*Fragaria* sp.) is conditioned by a number of factors, including photoperiod. Sensitivity to photoperiod varies among strawberry genotypes, which are generally divided into three categories: short-day, everbearing, and day-neutral. The genetic basis for these variations is not known, but existing model plant systems such *Arabidopsis thaliana* and rice reveal a well-conserved network of genes and proteins governing the perception of photoperiod and the regulation of floral initiation. A number of these genes were identified and characterized in strawberry and other relatives in the family Rosaceae, and their expression in strawberry under long and short photoperiods assayed. Expression profiles suggest that while many of the critical genes governing photoperiodic flowering are conserved between strawberry and model species, their expression and relationships to one another are unlike those of any previously characterized plant species.

CHAPTER 1
INTRODUCTION

The initiation of flowering is among the most important developmental transitions a plant makes, second perhaps only to germination. Mistimed flowering endangers the reproductive potential of plant in a number of ways. A plant that flowers too early or too late may find the seasonal conditions not conducive to seed development, or yield seeds that germinate at points in the season that make seedling survival difficult or impossible. In cross-pollinated species even a difference of a few days may be adequate to render reproduction impossible, putting a plant out of syncronization with others of its species. Any of these difficulties has the potential to render an individual an evolutionary dead end.

Because of this necessity, plants have developed an extraordinary regulatory network allowing the fine tuning of the transition to flowering by a range of environmental and internal factors. In the model eudicot Arabidopsis, these factors have been shown to include the environmental inputs of photoperiod, light quality, and temperature, and internal pathways controlled by the hormone gibberellic acid and autonomous factors such as developmental age.

At the molecular level, each of these inputs represents separate elements of genetic pathways that converge at a few floral integrators to regulate flowering through a common set of meristem identity genes. These integrators convert the complex and heterogeneous inputs from the various environmental and internal cues into a decision to flower, influencing a choice made in a small cluster of meristmatic cells, and driving development from a vegetative to reproductive state through the activation of genes that remodel cell fate. Key among these floral integrators is *CONSTANS* (*CO*), which serves to tie the perception of photoperiod with that of the spectral quality of light received.

Flowering in cultivated strawberry (*Fragaria ×ananassa* Duch.) has historically received a great deal of attention from researchers, for two primary reasons. First, strawberry is an attractive platform for flowering research because despite a narrow genetic base (Sjulin & Dale, 1987) it exhibits at least three distinct and ranging flowering habits: junebearing (short day), day-neutral (photoperiod insensitive), and everbearing (often referred to as "long day" but more accurately another distinct form of photoperiod insensitivity). These clear phenotypic delineations in such a genetically homogeneous background suggests fairly simple genetic difference between markedly contrasting flowering habits.

Secondly, in an increasingly global market where fruit is readily transported not only across countries but around the world, farmers depend on a precise understanding of flowering behavior to time harvest to coincide with the best market windows in order to obtain the best price for their fruit. In an era of narrowing profit margins, this timing may be the difference between a profitable crop and a loss.

CHAPTER 2
LITERATURE REVIEW

The genus *Fragaria*

All strawberry species belong to the genus *Fragaria,* and are members of Rosaceae, a family that contains a large number of economically significant crops, primarily fruits such as apple (*Malus domestica* Borkh.), pear (*Pyrus* sp.), stone fruits (*Prunus* sp.), and brambles (*Rubus* sp.), but also ornamentals such as rose (*Rosa* sp.). Strawberries belong to the Rosoideae subfamily, which includes the genera *Rubus, Rosa, Potentilla*, and *Duchesnea*. Authorities vary somewhat on the exact number of strawberry species, but most recent authors list about twenty species (Hancock and Luby, 1993; Folta and Davis, 2006). Native strawberries occur in much of North America, Europe, and Asia, as well as parts of South America, and Hawaii (Darrow, 1966; Hancock and Luby, 1993).

Cultivated strawberry, *F.* ×*ananassa* Duch., is a species of relatively recent hybrid origin, being derived from the accidental hybridization of two New World octoploid species, *F. virginiana* Duch. and *F. chiloensis* (L.) P. Mill., beginning in France some time in the late 1700s (Darrow, 1966). Although the polyploid nature of the crop and its hybrid origin have assured that the species contains some genetic diversity, the actual germplasm base used is relatively small, with only 53 founding clones (Sjulin and Dale, 1987) and 17 initial cytoplasm donors (Dale and Sjulin, 1990) contributing to the cultivated varieties. Some recent introgressions from the wild have been made, most notably from a selection of *F. virginiana* ssp. Duch. var. *glauca* (S. Wats) Staudt, but in general very little new genetic material has been added since the early years of strawberry breeding (Darrow, 1966).

Polyploidy in Strawberries

As has been previously mentioned, the cultivated strawberry is an octoploid (2n=8x=56). The majority of strawberry species, however, are diploids, and there exist tetraploid and hexaploid taxa as well (Folta and Davis, 2006). Considerable evidence suggests that the cultivated strawberry is an alloploid, with either three or four distinct subgenome types. The earliest model, that of Federova (1946), proposed a composition of AABBBBCC, but this was replaced with AAA′A′BBBB when Senanayake and Bringhurst (1967) found evidence of partial homology between the A and C genomes. Mounting evidence of diploidization (Byrne and Jelenkovic, 1976; Arulsekar, et al. 1981) prompted Bringhurst (1990) to propose a fully diploidized model: AAA′A′BBB′B′.

The origins of the component genomes have long been a subject of debate. As early as 1926, Ichijima suggested that one of these might be derived from *F. vesca*, based on cytogenetic observations of *F. virginiana* (8x) x *F. vesca* L. (2x) hybrids, in which the formation of seven bivalents was noted. Further data, including cytology (Senanayake and Bringhurst, 1967) and sequence analysis of the chloroplast trnL-trnF region (Potter et al., 2000), the nuclear sequences ITS (Potter et al., 2000) and polygalacturonase inhibitor protein (PGIP) (Chapter 5 of this work), have strengthened this view, and the A genome is now widely believed to be derived from *F. vesca* (Folta and Davis, 2006).

The origins of the other genomes have been less clear. The study of ITS and trnL-trnF sequences implicated *F. nubicola* as a possible A-type genome donor, in addition to *F. vesca* (Potter et al., 2000). Recent work by DiMeglio and Davis (unpublished but cited in Folta and Davis, 2006) examining the alcohol dehydrogenase locus implicates two other species, *F. mandshurica* Staudt and *F. iinumae* Makino. Potter et al. (2000) and Harrison et al. (1997) found

F. iinumae to be the most distantly related of all other *Fragaria* species examined, and as such may represent the ancestor of one or both of B and B' genomes. Unlike most diploid species, which possess at least a degree of compatibility with other diploid *Fragaria* (Dowrick and Williams 1959), *F. iinumae* is not compatible with *F. vesca* (Bors, 2000; Sargent et al., 2004b), and displays a karyotype distinct from the other diploid species (Iwatsubo and Naruhashi, 1989, 1991), which may account for the failure of B genome chromosomes to pair with those of the A genomes. *F. mandshurica*, on the other hand, is a newly described diploid species (Staudt, 2003) not included in previous studies. It is however thought to be related to the tetraploid species *F. orientalis* Losinsk., to which it bears a strong resemblance. Potter et al. (2000) had placed *F. orientalis* in the A clade, and thus *F. mandshurica* may represent A' genome in the octoploid species.

Flowering Habits in Strawberry

Although many authors (Fuller, 1897; Fletcher, 1917) had suggested the presence of environmental influences on flowering in strawberry, Sudds (1928) was perhaps the first to note the specific effects of photoperiod and temperature on flower bud induction in strawberry. Soon after, Darrow and Waldo (1929, 1930, 1933) conducted an exhaustive series of experiments, classifying over a hundred octoploid strawberry genotypes as flowering under either short or long days, finding the groups to generally coincide with what were already termed "June bearers" and "ever bearers".

Short-Day Strawberries

It is believed that the natural state of most strawberry species is as a short day (SD) plant (Darrow, 1966), though there is considerable variation in senstivity. It appears that the earliest cultivated octoploids all flowered under short days (Darrow, 1966), although the limits of the required photoperiod vary considerably between genotypes. In general, most SD genotypes form

flower buds under photoperiods of less than 14 h, which was first suggested by Darrow (1936). Three major factors seem to dictate response to photoperiod: genotype, temperature, and chilling.

Genotype effects on photoperiodic flowering in SD strawberries

Darrow concluded that photoperiods of 9.5 to 13.5 h induced the greatest number of flowers in SD cultivars, with an optimum around 12 h (1936). While still generally true, further studies and perhaps continued progress in breeding have widened this range somewhat. On one end of the range, some authors (Izhar, 1997; Faedi et al., 2002) have delineated a class of strawberries they refer to as "infra short-day." These plants flower under longer day lengths (typically 13.5-14 h photoperiod) and with little chilling requirement, but seem to represent merely an extension of the existing spectrum of sensitivities already known in SD plants, rather than a distinct flowering habit. In other genotypes, photoperiods of considerably less than 12 h are required for optimal flowering. In a study by Heide (1977) 'Abundance' demonstrated peak flowering under a 10 h photoperiod, the shortest tested in the study. A number of studies have shown robust flowering under 8 h photoperiods in some cultivars (Sonsteby, 1997; Leshem and Koller, 1964; Barger et al. 1997; Moore and Hough, 1962). Many early researchers compared short and long day lengths in terms of natural light versus natural light plus an artificial extension of day length, making it difficult to assign specific photoperiods.

It is interesting to note that while SD photoperiods are needed for optimal floral induction, they may actually delay floral development. Several studies of SD cultivars have shown that continuing SD photoperiods may actually slow the development of previously initiated buds compared to longer photoperiods (Moore and Hough, 1962; Durner and Poling, 1987). Consequently, Salisbury and Ross (1992) consider strawberries to be SD for purposes of flower induction, but LD plants for flower development. A similar relationship for temperature has also been noted (see below).

Remontant Strawberries

Alpine strawberries

The first major exception to this flowering pattern noted was the alpine strawberry, *F. vesca* var. *semperflorens* Duch. Found at several places in the European Alps, these represent a mutation of the common diploid European wood strawberry, and were among the earliest types to be cultivated. Though first mentioned as early as 1553, they gained prominence in the mid 1700's when they were introduced into France and England, possibly from Turin, Italy (Duchesne, 1766). In 1811, a runnerless form was obtained by Labaute (Darrow, 1966). Now only occasionally cultivated, these varieties are of only minor commercial importance (Darrow, 1966), although in the early nineteenth century a white-fruited variety was extensively cultivated in Quebec (Fletcher, 1917). Fairly little work has been done to characterize the floral physiology of these varieties, and the fact that many workers have studied unnamed genotypes that are either seed-propagated or even collected directly from the wild (Chabot, 1978) makes it difficult to consistently track genotype-specific differences. Sironval and El Tannir-Lomba (1960) found that their selection of *F. vesca* var. *semperflorens* required days longer than 12 h to form flower buds, and that SD treatments inhibited flowering in plants that had previously reached the flowering stage under LD conditions, though this appears to be the exception, not the rule (Darrow, 1966). As in other strawberries, temperature has a significant effect, with moderately low temperatures favoring both reproductive development and biomass production (Chabot, 1978).

"Everbearers"

Relatively quickly after the introduction of octoploid cultivars, however, so-called "everbearing" (EB) strawberries were identified (Darrow, 1966). Such genotypes initiate flowering most heavily under the long days of summer, and often on unrooted or newly rooted

runners, resulting in what is in most locations primarily a fall harvest, rather than an early summer one, or in some cases two distinct crops.

These genotypes appear to trace to a few distinct sources within cultivated types. suggesting spontaneous mutation of a single gene. Development of such types took place independently in both North American and Europe, but very little crossover has ever occurred between these two sources of the trait. The first North American EB cultivar described was possibly 'Oregon Everbearing,' obtained in 1882, although Fletcher (1917) describes an everbearing selection of what was likely *F. virginiana*, found in Ohio in 1852. The first successful everbearer, however, was likely 'Pan-American,' an apparent sport of the June bearer 'Bismarck' found in 1898 (Fletcher, 1917). 'Pan-American,' in turn, became the source of the trait for a number of other successful everbearing cultivars, including 'Progressive' and 'Rockhill' (Darrow, 1966). This last selection is the source of the trait in the proprietary everbearing cultivars developed by Driscoll Strawberry Associates (Darrow, 1966). Other sources of the trait in North America include 'Gem,' a sport of 'Champion' introduced in 1933 that proved quite popular for several decades. That the trait is the result of instability at a single locus is supported by documented reversion to short day flowering in the case of 'June Rockhill', a short day mutant of the everbearer 'Rockhill' (Darrow, 1966).

European octoploid everbearers, called "*remontant*" by the French, may have had their origin somewhat earlier. Unlike the American sources of the trait, which can largely be traced to mutations identified in cultivated plants, the source of the European everbearers is unclear. Although claimed at the time to be hybrids of large-fruited octoploids with the everbearing Alpine diploids (Fletcher, 1917), it seems doubtful that this was truly the case. First, such hybrids of *F. vesca* with the octoploids are difficult to make (Yarnell, 1931; Mangelsdorf and

East, 1927) and even more difficult to restore to adequate fertility, and secondly the inheritance of the trait in the octoploids seems to be different than in Alpine strawberries (Brown and Wareing, 1965; Clark, 1937). More likely, accidental cross-pollination resulted in seedlings of pure *F.* ×*ananassa* pedigree, among which was selected the original everbearer. Some, such as 'Louis Gauthier,' a double-cropping French cultivar, may have been simply SD genotypes with unusually permissive photoperiod or chilling requirements (Fletcher, 1917). Richardson (1914) suggests that the first true European octoploid everbearer was 'Gloede's Seedling,' introduced in 1866, and this may be the source for most or all European everbearers that followed (Ahmadi et al., 1990). 'Mabille' and 'l'Inepuisable,' considered by some (Fletcher, 1917) to be pure Alpine strawberry, followed this, but none were commercially successful. A French priest, Abbe Thivolet, was among the first to breed for this trait, and after more than a decade of breeding both octoploids and diploids (Fletcher, 1917) introduced a series of everbearers, culminating in 'St. Joseph', which Darrow (1966) refers to as the "first true large-fruited everbearer." Although a considerable improvement over the preceding everbearers, 'St. Joseph' and Thivolet's later 'St. Antoine de Padoue' were not especially productive, but spurred the development of later improved types. While EB plants derived from this source remain in production today, this source has contributed very little to American breeding.

Although they have been called "long day" cultivars, these varieties lack the characteristic behaviors associated with true long day plants, in the sense that they do not appear to be regulated by the length of the dark period, and do not react identically to a short day with a brief night break of light as they do to long photoperiods (Dennis et al., 1970; Durner et al. 1984), nor is flowering inhibited by SD (Durner et al., 1984). Rather, these genotypes seem more dependent on the total amount of light received. Dennis et al. (1970) found no difference in flower

formation in the everbearing cultivar Geneva grown under 12 h photoperiods versus those grown under a 10 h photoperiod with a 2 h night break, a treatment which often inhibits flowering in SD plants (Downs and Piringer, 1955; Piringer and Scott, 1964). Marked increases, however, were seen with 18 and 24 h photoperiods. Additionally, they saw a significant effect of light intensity when increased from 1200 to 2400 f-c on the 18 and 24 h photoperiod treatments (increases were also seen on the 12 h and 10 + 2 h treatments, but these were not statistically significant). This study and the later study by Durner et al. (1984) both noted similar levels of flowering for both 12 h photoperiods and 10 + 2 h night break treatments, suggesting that amount of light, not duration of either darkness or light, is the critical factor.

"Day-neutrals"

The third distinct flowering habit is day-neutral (DN). Observers of wild American octoploids had noted that many plants of *F. virginiana* subsp. *glauca* (then called *F. ovalis*) flowered in the summer and fall when most wild and commercial short day cultivars had stopped (Darrow, 1966). Researchers with the USDA evaluated a large number of *F. virginiana* subsp. *glauca* selections in the 1930s and 1940s, and a many were included in breeding efforts at the time. A number of everbearing varieties resulted from these efforts, including two, 'Arapahoe' and 'Ogallala,' (Hildreth and Powers, 1941) that are still in existence today. Whether the trait expressed in these cultivars was the older EB trait or the DN trait derived from *glauca* is unknown, as sources of both are present in the pedigrees, the strong tendency of most remontant seedlings of 'Arapahoe' and 'Ogallala' to fruit in their first year is distinct from those other EB parents (Ourecky and Slate, 1967), and more typical of DN genotypes (Ahmadi et al., 1991). Regardless, the introduction of the DN trait that has had the largest impact came in the 1970's, when the University of California breeding program, under Royce Bringhurst and Victor Voth, utilized a single selection of *F. virginiana* subsp. *glauca* from the Wasatch Mountains in Utah to

introduce this habit into commercial strawberries (Bringhurst and Voth, 1984). Strawberries derived from this source constituted a majority of the cultivars planted in California in 1999 (Hancock, 1999).

These were distinct from the everbearing habit in that the plants were truly insensitive to the length of the photoperiod received, and fruit at nearly the same rate over a broad range of photoperiods (Durner et al., 1984; Ahmadi et al., 1991). Day-neutral cultivars offered improved heat tolerance and longer harvest seasons compared with the earlier everbearers, but runner poorly and are difficult to propagate (Durner et al., 1984). Although day-neutrals appear to differ from older everbearing types, many researchers have failed to distinguish between them, and in some cases use the terms interchangeably, resulting in confusion. For the purposes of this work, "day-neutral" (DN) will refer to the trait derived from *F. virginiana* ssp. *glauca,* while "everbearing" will refer to that derived from 'Pan-American', 'Gloede's Seedling,' or other older sources, and the generic "remontant" will be used to refer to cases where both are implied or the author has not been clear in distinguishing.

Other flowering habits

Arguably a fourth flowering habit, poorly characterized at this time, is the amphiphotoperiodic behavior exemplified by the *F. chiloensis* selection CHI24-1 (Yanagi et al. 2006). This genotype behaves as a standard SD plant under most conditions, with long photoperiods inhibiting flowering, but initiates flowers under continuous lighting as well. Although many will cease flowering altogether (Thompson and Guttridge, 1960), a few cultivated SD strawberry cultivars may fall into this category, as both 'Sparkle' (Collins and Barker, 1964) and 'Sweet Charlie' (unpublished observations) have been observed to flower under such conditions as well, but thus far little research has been done on this phenomenon.

There has been much interest in recent years in finding novel sources of remontancy, and workers have identified a number of wild accessions that seem to display varying degrees of photoperiod-insensitivity or continual flowering, mostly among *F. virginiana* (Sakin et al., 1997; Hancock et al., 2001, 2002; Serçe and Hancock, 2005a, b). These new sources have not yet been adequately characterized to determine whether they fall neatly into the DN or EB categories, or whether they represent truly novel mechanisms. Inheritance studies by Serçe and Hancock (2005a) suggest that there may be multiple genetic mechanisms at work.

Factors Affecting Expression Of Photoperiodic Flowering

Temperature Effects On Flowering

A relationship between temperature and flower initiation was established by early workers, and was highlighted in the large studies of flowering by Darrow and Waldo (1929, 1930, 1933). Research has also shown that lower temperatures may permit flowering under longer daylengths even in normally SD cultivars (Darrow and Waldo, 1934; Darrow, 1936). Hartmann (1947b) concluded that this was the reason that cultivars grown in the coastal district of California near Watsonville, where the mean temperature is 17°C, were able to flower and fruit through the long days of summer, while the same cultivars planted inland near Sacramento, where the mean temperature was 23°C, behaved as strict short day plants and produced only a spring crop. In greenhouse studies he found that plants of four cultivars important to the region all flowered under long days at 17°C, but none flowered at 23°C. Three of the four same genotypes flowered freely under both temperatures under short day conditions, while a fourth, 'Fairfax,' failed to flower at all under the higher temperature, even under 10 h days. Even everbearing cultivars under long days are inhibited by high temperatures, as are day-neutral types, though in general the critical temperature is higher for DN cultivars (Durner et al., 1984; Manakasem and Goodwin, 2001). Heide (1977) found two of four Scandinavian SD cultivars still flowered under

photoperiods of 14, 16, and even 24 h when grown at 12°C or 18°C, though flowering was substantially decreased under the longer photoperiods. The effects were highly dependent on genotype, however, particularly in the middle of the temperature range, a consistent finding of such studies. In some cases cultivars that are considered to be short day in some locations may behave as remontant at other locations . 'Climax', a British selection, behaved like a SD plant when grown in the United States, but as a two-crop variety in England where temperatures are coller and summer days are longer (Downs & Piringer, 1955).

Much like SD photoperiods, which are necessary to develop flower buds, but inhibit the development of flower trusses, low temperatures allow for flower induction under long photoperiods, but may not be optimal for fruit production, because lower temperatures slow development of the flower trusses (Darrow, 1966). Le Miere et al., (1996) found optimum temperatures for truss development to be about 19°C in 'Elsanta'. Temperatures that fluctuate diurnally seem to be more effective in promoting flower development than continuous temperatures, even at the same average temperature (Hartmann, 1947a). Such thermoperiodic rhythms have been shown to have similar effects in other species (Seneca, 1974; Alvarenga and Válio, 1989) and may allow for more robust entrainment of the circadian clock (Salomé and McClung, 2005).

Vernalization Requirements

Temperature also plays a second role in the expression of flowering, by conditioning the plant for flowering before the season begins. A certain amount of chilling, generally defined as time between 0°C and 7°C, may be required to break bud dormancy and proceed with the normal cycle of development, though the extent of chilling required is highly cultivar-dependent (Piringer and Scott, 1964; Voth and Bringhurst, 1970; Durner and Poling, 1986; Darnell and Hancock, 1996). Increased chilling has been shown to correlate with increases in leaf area and

number, petiole length, and runner initiation (Guttridge 1969; Bringhurst et al., 1960; Piringer and Scott, 1964; Braun and Kender, 1985; Lieten, 1997). Chilling has been shown to promote both vegetative and reproductive development (Darnell and Hancock, 1996). Durner and Poling (1987) found chilling to enhance vegetative growth and reduce flower induction while promoting floral differentiation. Non-chilled plants produce fruit of smaller size (Harmann and Poling, 1997) and lower quality (Bringhurst et al., 1960) than do the identical genotypes with adequate chilling.

Because of the related decrease in flower induction, however, chilling beyond the required amount is of no advantage and can even markedly reduce the number of flowers. Piringer and Scott (1964), for example, saw substantial decreases in flower number on 'Marshall' with increasing chilling.

Juvenility and Plant Age Effects

Juvenility may also play a role in the expression of photoperiod-sensitivity. In general, young plants devote more resources to vegetative than reproductive growth, and may not flower at all during this portion of their life cycle. This allows a plant to attain sufficient size to support fruit and seed development before flowering (Thomas and Vince-Prue, 1984) Ourecky and Slate (1967), working with the EB trait, noted that when populations were retained for a year and scored for a second season, more plants were determined to be everbearing (a finding supported by unpublished worked cited in Scott & Lawrence (1975)), casting a degree of doubt on earlier work that utilized only first year data. However, Ahmadi et al. (1990) found that DN progeny generally flowered within four months of germination. While not an issue for plants in cultivation, this effect may have serious impacts on the ability of breeders to make selections based on flowering habit. Ito and Saito (1962) found that older plants responded more robustly to

both photoperiod and temperature. This may have been in part a function of plant size, although Hartmann (1947a) found that a single intact leaf was enough to perceive inductive photoperiods.

Growth Pattern Differences Between Plants Of Differing Flowering Habits

In addition to the differences in flower production, strawberries of differing flowering habits also differ in terms of plant architecture and growth patterns. The production of runners varies considerably among SD, DN, and EB genotypes. Runner initiation is affected by photoperiod and temperature as well. In SD cultivars, there appears to be a balance between runners and inflorescences ranging from almost no runners and many flowers under optimal SD conditions, through an intermediate area where flowers and runners coexist, and then solely runnering under the longest days. SD plants will runner in response to short days with a night break, and they tend to shift from reproductive to vegetative growth under high temperatures (Piringer and Scott, 1964; Durner et al., 1984). Interestingly, DN cultivars, while flowering in a photoperiod insensitive manner, seem to retain a sensitivity to photoperiod in respect to runnering, with the number of runners increasing under long days, night break, or high temperature conditions. This is in contrast to EB types, which do not show photoperiodic or temperature changes in runner initiation (Durner et al., 1984). In many, though not all cases (Durner et al., 1984) EB genotypes runner very little, which may be a barrier to efficient propagation.

Nicoll and Galletta (1987) also noted differences in plant architecture between DN, EB, and SD plants. Under long days, the main axis of SD and weakly DN plants remains vegetative and most axillary buds develop as runners, while under short days, the main axis terminates in an inflorescence and an upper axillary bud develops as a branch crown. In EB plants, under all photoperiods, most or all axillary buds that develop do so as branch crowns, rather than runners, and it is the termination of these crowns with inflorescences that results in most flowering,

though the main axis will occasionally terminate in a flower and continue growth from a side shoot as in SD plants. In DN plants, by contrast, the growth is characterized by very low rates of branch crown formation, with upward growth quickly terminated by a terminal inflorescence and continued from an upper bud, with reduced development of leaves, runners, and branch crowns. Interestingly, these growth habits closely parallel those seen in *F. vesca* by Brown and Wareing (1965), but in that case were found to associate closely with the runnering locus, or a gene close by it, rather than the seasonality locus, described below. The runnerless gene from 'Baron Solemacher' conveyed a growth pattern like that observed in DN plants, while runnerless plants descended from 'Bush White' had a many-crowned habit like that of EB octoploids, and the wild type *F. vesca* was similar to the SD octoploids.

Inheritance Of Flowering Habit In Strawberry

Inheritance of photoperiod insensitivity has been described in a number of species. Most commonly, it is a single gene trait, conferred by either a dominant allele, as in rice (*Oryza sativa* L,) (Chandrartna, 1953), sweet pea (*Lathyrus odoratus* L.) (Ross and Murfet, 1985), jute (*Corchorus* sp.) (Joshua and Thakare, 1986), and tetraploid Sea Island cotton (*Gossypium barbadense*) (Lewis and Richmond, 1960), or a recessive allele, as in diploid upland cotton (*Gossypium hirsutum* L.) (Lewis and Richmond, 1957), cucumber (*Cucumis sativus* L.) (Dellavecchia and Peterson, 1984), or okra (*Abelmoschus esculentus* (L.) Moench) (Wyatt, 1985). In a few instances, the inheritance has been shown to be a more complicated mechanism, such as the two dominant alleles at separate loci responsible in some hexaploid wheat (*Tritcum aestivum* L.) (Maystrenko and Aliev, 1986) or the three genes believed to be involved in sesame (*Sesamum indicum* L.) (Kotecha et al. 1975).

Brown and Wareing (1965) demonstrated that seasonality in *F. vesca* is conveyed by a single gene, which they designated *S* (referred to as *SFL* by some later authors (Albani et al.,

2004)), with the perpetual flowering habit displayed by the homozygous recessive. They further demonstrated that the trait was independently inherited from the non-runnering habit, which proved to be another recessive trait at a different locus. Federova (1937) may have identified seasonality in *F. vesca* as a recessive trait earlier, but it is unclear with which species he was working. Ahmadi et al. (1990) expanded on this by observing that the California *F. vesca*, which differs significantly from the European *F. vesca* morphologically, appears to have two additional genes conveying photoperiod sensitivity, yielding a 1:63 ratio in the F_2 of a cross between Alpine *F. vesca* and local California plants.

Richardson (1914) conducted some of the first studies of the inheritance of the EB trait. Using 'St. Antoine de Padoue' and 'Laxton's Perpetual', both derived from 'St. Joseph', as sources of the trait, he found ratios of 1:1 and 3:1, suggesting a simply inherited character.

A considerably different model was that of Clark (1937), who investigated the expression of the EB trait from 'Mastodon', an everbearer derived from 'Pan-American', in breeding populations grown in New Jersey. In general, there was a strong trend towards producing approximately one third everbearers in crosses of EB with non-everbearers (presumably SD), with an average of two-thirds in crosses of EB x EB, whereas Mastodon selfed yielded 80% EB, though in a small population. Clark also cites an earlier EB x EB cross, reported by Macoun (1924), which yielded slightly less, 56.29% EB. In nearly all cases, crosses of SD x SD, in which one of the parents had one EB parent, yielded no EB progeny. There were, however, exceptions. One SD plant with an EB parent, N.J. 220, did give some EB offspring in one cross. Additionally, two everbearers, N.J. 1 and N.J. 8, produced very low numbers of everbearers in their progeny in crosses with non-everbearers (0% and 8.8%, respectively) and when selfed (0% and 11.9%, respectively). Clark concluded that the results suggested a complex,

polygenic inheritance, but with evidence that the character is largely, though not wholly, conveyed by a major, dominant allele, possibly modified by other genes.

Later work attempted to clarify this model, although with limited success. Powers (1954) performed a partial diallel crossing of three EB and seven SD genotypes and developed a three locus model with four dominant alleles, *A', A, B,* and *C*. conveying the everbearing trait with varying strength, ranging from *A'>A>B>C*. Any of these alone was theorized to be inadequate for expression of the trait, as was *aaB_C_*, but *A'* or *A* plus a dominant allele at either of the other loci resulted in the everbearing character. He also theorized that as many as four recessive genes account for the presence of EB plants in the SD x SD progenies.

Although mostly adequate to explain the observed data (though 5 of his 49 families do exhibit high chi-square values with this model), Powers' model assumed that all seven of the SD parents in his study were of the same genotype, *aaBbCc*. Unless there is a significant unknown selective value to the heterozygote at the B and C loci, it seems exceedingly unlikely that all three unrelated short day genotypes would be double heterozygotes.

Two confounding factors may have been at work here. First, the everbearing parents used are primarily breeding selections, so the source of the trait cannot be readily ascertained. Since the Cheyenne USDA breeding program was utilizing *F. virginiana* ssp. *glauca* in addition to EB cultivars as parents, it is possible that Powers' observations are the result of a mingling of DN and EB sources. Secondly, Powers used the production of flowers in July, August, or September as criteria for EB. In light of the fact that Powers' experiments were conducted in the field at Cheyenne, Wyoming, it may be that low temperatures allowed flowering to continue even during the long days of summer. Mid-summer day length in Cheyenne is slightly less than 15 h, whereas the 1971-2000 mean monthly temperatures for Cheyenne in June, July, August, and September

were 16.4, 19.8, 18.8, and 13.6°C, respectively (National Climatic Data Center, 2001). Heide (1977) found that at 18°C, three of four SD cultivars still initiated at least some flowers under 14 h days, and thus Powers may have, in fact, been observing everbearing behavior in non-everbearing genotypes. Later studies may have been affected similarly.

Ourecky & Slate (1967) examined 46 progenies and suggested complementary dominant genes segregating in an octoploid manner. This is somewhat at odds with later work suggesting that octoploid strawberries are diploidized (Arulsekar et al. 1981), despite recent hybrid origin. More recent work by Sugimoto et al. (2005) found a 1:1 ratio of EB : SD in a cross of 'Ever Berry' (EB) x 'Toyonaka' (SD), as well as in its reciprocal, whereas 'Ever Berry' selfed gave 3:1 and 'Toyonaka' selfed gave all SD progeny. This is in line with earlier work using 'Ever Berry' as a parent (Monma et al., 1990; Igrashi et al., 1994), and implies a simple monogenic dominant inheritance.

Although most authors have considered all sources of the EB trait to be genetically identical, it may be worth noting that these Japanese studies, as well as Richardson's early studies (1914), the only studies to show clear monogenic ratios, have used cultivars that probably carry the everbearing trait derived from the European source (or sources). The studies which have obtained multigene or confusing inheritance ratios have all used American sources of the trait: Clark (1937), used 'Mastodon', derived from the 'Pan-American' source; Ourecky and Slate (1967) used a wide range of cultivars derived from either 'Pan-American' or 'Streamliner' (a seedling of unknown pedigree); and Powers (1954), as previously mentioned, may have been using a mix of EB and DN parents. Thus the traits, while similar, may have slightly different genetic mechanisms, or the American sources possess contain modifying genes not found in the European cultivars.

Characterization of the inheritance of the day-neutrality trait has been clouded by the fact that studies have indiscriminately included "everbearers" from both the 'Pan-American' source and true day-neutrals from the *F. virginiana* ssp. *glauca* source. At first, day-neutrality was thought to be the result of a single dominant gene. Ahmadi et al. (1990) found this in an inspection of nearly 30,000 progeny of crosses between day-neutral and short day plants over the course of five years. These results firmly agree with a single-gene hypothesis, showing significant deviation from the model in only one family during one year. Importantly, unlike early work on everbearers, there were no seedlings that expressed the trait among progenies from SD x SD. Although the study clearly deals primarily with the *glauca* source, it is not clear which remontant cultivars were used and whether all derive from *glauca*. However, their results differ significantly from later studies that did include EB from the 'Pan-American' source, which suggests that all four of the DN cultivars used are derived from *glauca*.

Serçe and Hancock (2005a) also studied the inheritance of day-neutrality, looking at both cultivated DN and EB cultivars, and apparent novel sources of remontancy found in wild *F. virginiana*. Segregation ratios varied widely, apparently suggesting inheritance more complex than a single gene model. A similar study by Shaw and Famula (2005) using 45 cultivated genotypes thought to derive their photoperiod insensitivity from the *F. virginiana* ssp. *glauca* source, though not identified in the paper, found strong evidence for the presence of a major dominant locus for day-neutrality, with putative homozygotes flowering more robustly under LD conditions than heterozygotes. However, it was noted that even homozygous DN plants were not wholly true-breeding for the trait, suggesting either the effects of other minor loci or deviations from diploid inheritance.

The system used for scoring progenies may have had a significant impact on the results of such studies. A primary difference between Richardson's (1914) studies and many of those that followed is that Richardson considered everbearers to be individuals that continued flowering through October, rather than stopping at the end of the summer. Ahmadi et al. (1990) considered this a more accurate identification of remontant genotypes, but theirs appears to be the only other study to have done this, as all others reviewed here continued ratings only into August or mid-September. Serçe and Hancock (2003) evaluated five methods of scoring populations for remontancy, including flowering within 100 days of germination, flowering before a specific date in the field, flowering under both long and short days in greenhouse or field, and flowering on newly formed runners in the field. Scoring by flowering within 100 days of germination was not a good predictor of remontant flowering in the field, but greenhouse observations, if conducted over the course of an entire season, were well correlated to field performance.

Molecular Markers For Flowering Habit

The ability to screen seedlings for flowering habit at a very early age would be of great benefit to breeders, saving the time and resources required to plant out and evaluate seedlings lacking the desired habit. A number of attempts have been made to develop such a system, but none has seen wide application in breeding.

Albani et al. (2004) used inter-simple sequence repeat (ISSR) markers to identify three DNA products associated with seasonal flowering in 1,049 plants of a *F. vesca* ssp. *vesca* (*SFL/SFL*) x *F. vesca* ssp. *semperflorens* (*sfl/sfl*) BC_1 population, and successfully converted these to sequence characterized amplified region (SCAR) markers. Two of these markers were linked to the seasonal flowering locus at 1.7 and 3.0 cM, while a third, SCAR2, was mapped to the same location as *SFL*. This represents the most tightly associated marker for flowering habit yet developed in *Fragaria*, but while this work constitutes an important research tool, the impact

of these markers on practical breeding has been limited because of the differences between this trait and the DN and EB traits of the cultivated octoploid, as well as the lack of commercial importance of *F. vesca*.

Kaczmarska and Hortynski (2002) identified a single randomly amplified polymorphic DNA (RAPD) marker through bulk segregant analysis of a small F_1 population, segregating 1:1 for remontancy; however, no attempt was recorded to discern how closely linked this marker was with the trait or how reliable it was across other progenies.

The previously cited study by Sugimoto et al. (2005) attempted to identify RAPD markers linked to the EB trait. Five were identified; however the linkages, ranging from 11.8 to 24.3 cM, were rather weak. These weak linkages, coupled with the difficulties sometimes encountered when trying to reproduce RAPD markers (Paran and Michelmore, 1993), may limit the practical use of these markers.

Molecular Control Of Flowering In Arabidopsis

Although the effects of genetic variation on flowering, even allelic variation at a single locus, could be clearly seen in many species, the underlying system often proved complex, making it difficult to elucidate the roles of individual genes clearly. Among the first steps towards such an understanding was made through the development of mutant lines of *Arabidopsis thaliana,* (a long day (LD) annual species), displaying aberrant flowering patterns. Reinholz (1945) noted differences in flowering among irradiated Arabidopsis seedlings, and Rédei (1962) followed up on this work by treating imbibed Arabidopsis seeds with X-rays. He selected four mutants that exhibited altered timing of flowering, designating them *constans* (*co*), luminidependens (*ld*), and *gigantea* (*gi-1*, *gi-2*). All of these lines flowered significantly later than the wild type control under long days, although the *co* plants flowered slightly faster under short photoperiods.

Subsequent work has shown *co* to be an important element of a complex network of regulatory pathways governing flowering which are linked specifically to the perception of photoperiod. There are three distinct parts to photoperiod perception in Arabidopsis: photoreceptors that perceive light, an internal oscillator that approximates a 24 h cycle, and the output path to the meristem identity genes involved in flower initiation (Simpson, 2003). CO itself lays at the junction between the inputs of light quality and photoperiod on one side, and on the other side a series of genes, such as *FT* and *SOC1*, directly upstream of the meristem identity genes. A simplified diagram of this network is shown as Figure 2-1.

Components Upstream of *CO*

The perception of photoperiod begins with photoreceptors. Two groups, the phytochromes (*PhyA, B, D,* and *E*) and the cryptochromes (*Cry1* and *2*), react to red/far-red and blue light, respectively, to entrain the complex feedback loop of the plant's central oscillator (Millar, 2003). The central oscillator, in turn, produces a number of rhythmic outputs, including *CO* expression (Suárez-López et al., 2001). This rhythm regulates the base expression level of *CO*; however, *CO* protein abundance is greatly influenced by a number of factors. Key among these are the further effects of photoreceptors on transcript and protein stability. PhyB has been shown to promote the degradation of *CO* protein under red light early in the day, while in the evening phyA counteracts the effects of phyB and stabilizes CO protein when activated under far-red light, as do the cryptochromes under blue (Valverde et al. 2004). Another apparent blue light receptor, FKF1, has been shown to be required to produce the peak in *CO* transcript level at the end of the day required to trigger flowering in Arabidopsis (Imaizumi, et al. 2003). A member of the same family of proteins as CO, CONSTANS-LIKE9, also appears to repress expression of *CO*, though the mechanism is currently unknown (Cheng and Wang, 2005), as does the very similar CONSTANS-LIKE10 (Cheng and Wang, 2006).

Components Downstream of *CO*

CONSTANS acts directly on two major downstream flowering components, *Flowering Locus T* (*FT*) and *SUPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1)* (Samach, 2000). Both ultimately act to trigger a suite of meristem identity genes, (in fact many of the same genes), leading to flowering, and either may be adequate to trigger flowering (Samach, 2000), though this is disputed (Yoo et al., 2005). In addition to photoperiodic control through CO, SOC1 also responds to signals from the other pathways regulating flowering in Arabidopsis, the autonomous, temperature, and gibberellin pathways (Samach, 2000; Moon et al., 2003), and acts as an integrator of these independent inputs with the common set of meristem genes downstream. While *FT* and *SOC1* act largely in parallel in inducing flowering (Moon et al., 2005), *FT* has been shown able to activate *SOC1* to an extent itself (Yoo et al., 2005). Of the two, *FT* appears more strongly induced by the photoperiod pathway, while *SOC1* is under greater control of the autonomous pathway and regulated primarily by the gene *Flowering Locus C* (FLC) rather than by *CO* (Moon et al., 2005). In Arabidopsis, another gene, *TWIN SISTER OF FT* (*TSF*), which is very similar to *FT,* has also been shown to perform similar functions (Yamaguchi et al., 2005) although there is evidence of temperature effects on both genes' activities, suggesting that *TSF* is more active at low temperatures whereas *FT* is more active at higher temperatures (Blázquez et al., 2003)

Primary among downstream genes is *LEAFY (LFY)*, which is activated by *SOC1* and triggers a number of genes controlling floral development, including *AP1* (Nilsson et al., 1998). *FT*, however, bypasses *LFY* and activates *AP1* directly (Nilsson et al., 1998), as well as other meristem identity genes such as *SEPALLATA3 (SEP3)* and *FRUITFULL (FUL)* (Teper-Bamnolker and Samach, 2005).

Acting as a counterbalance to *FT* is *TERMINAL FLOWER1* (*TFL1*). *TFL1* acts to suppress the reproductive transition in the meristem, repressing expression of *AP1*. *TFL1* is a member of the same family of genes as *FT* and *TSF*, and Hanzawa et al. (2005) demonstrated that a change of a single amino acid residue in *TFL1* is adequate to shift its function to that of a promoter like *FT,* though the resulting protein is a weaker promoter of flowering than *FT*. *TFL1* expression is elevated in *CO*-overexpressor lines, and may in fact mirror *CO*'s promotion of *FT* expression to an extent (Simon et al., 1996). Loss of function *tfl1* mutants are early flowering and the inflorescences, unlike the indeterminate growth pattern of wild type Arabidopsis, end in a terminal flower which is often characterized by abnormal floral organs (Bradley, 1997).

The CONSTANS-LIKE Gene Family

CO is the best understood of a large family of genes, all of which share some basic characteristics. Specifically, all encode proteins which possess a highly conserved region of 129 amino acids called the CCT ("CO*,* COL*,* and TOC", after the three classes of genes that possess it) domain near the C-terminal end, as well as two zinc-finger type domains side by side at the other end, though the exact amino acid composition of these domains varies. There are also four small internal domains present in the canonical *CO* protein that are retained to some extent in various other members of the *COL* family. All are transcription factors, though their exact mode of action is still unknown.

Griffiths et al., (2003) characterized this family in Arabidopsis, rice, and barley, and found that three general groups were discernable in all three species, along with a fourth in the two monocot species. They were able to further subdivide this first group into three subgroups. The first of these, Group Ia, contains *CO* itself and a few closely related genes, specifically *COL1* and *COL2* in the case of Arabidopsis. These Arabidopsis genes have been shown to be unable to complement *co* mutants, and thus are not functionally interchangeable with *CO*, but do

have effects on circadian rhymicity of leaf movements and expression of *cab2* transcript (Ledger et al., 2001). Many species, including *Populus deltoides* and *P. trichocarpa* (Yuceer, 2002) and *Brassica nigra* (Lagercrantz et al., 2002), seem to possess multiple members of this subgroup, but it is not known whether these other members are functionally equivalent to *CO*. This study represents the first published documentation of Group Ia *COL* genes within the Rosaceae.

Group Ib *COL* genes are known only in monocots (Griffiths et al., 2003), but Group Ic genes, represented by *COL3/4/5* in Arabidopsis, are represented in nearly all species investigated (Jeong et al., 1999; Griffiths et al., 2003; Hecht et al., 2005). They differ from the Group Ia genes primarily in the middle region of the gene, where they lack the characteristic M2 and M3 domains (Griffiths et al., 2003). The role of these genes is poorly understood, but at least one, *COL3*, has been shown to have effects on photomorphogenesis, flowering, and inflorescence development (Datta et al. 2006). Zobell et al. (2005) found members of this family to be the only *COL* genes present in the primitive plant *Physcomitrella patens*, suggesting that they may be the most ancient representatives of the family. Two Group Ic genes from apple, possibly paralogs resulting from alloploidy in the species' distant past, represent the only *COL* proteins previously characterized in the Rosaceae (Jeong et al., 1999). Unfortunately, the authors did not take into account the diurnal expression pattern of many *COL* genes when documenting the expression of the genes, so it is difficult to derive much meaningful expression information from their work. Another Rosaceae Group Ic gene, designated *PrpCo,* was mapped in a peach-almond mapping population (Silva et al., 2005), but did not correspond to any known quantitative trait loci (QTLs) for flowering time in almond and was not further characterized.

Group Ib, Id, Ie, and IV *COL* genes appear to occur only monocots, but Group II and III genes are present in Arabidopsis and other dicots. The functions of these groups of genes are still

imperfectly known. Very little information on Group II genes appears to be available. A group III gene, *AtCOL9,* has been shown to play a role in flowering, apparently through the suppression of *CO*, and, as a result, *FT* (Cheng & Wang, 2005). Early work presented by the same authors also showed a related gene, *AtCOL10*, seems to have similar effects(Cheng & Wang, 2006), and both appear to be expressed in a circadian fashion. A Group III gene from perennial ryegrass, *LpCOL1*, has also been shown to be under oscillator control and is implicated in vernalization (Ciannamea et al., 2006).

Components of the Flowering Pathways are Conserved Among Species

In some respects, Arabidopsis, a long-day annual, might seem a poor model for flowering in strawberry, a normally short-day perennial in an entirely different taxonomic family. However, while there are no doubt critical differences in the physiology of these two species, mounting evidence suggests that the pathways regulating flowering are remarkably well conserved between taxa, even those separated from Arabidopsis by considerable evolutionary distance. The components of the photoperiod and floral pathways in rice, a short day monocot species, have proven nearly identical to and interchangeable with those of Arabidopsis (Izawa et al., 2003).

CO has been identified in a number of other species. Besides the model species of Arabidopsis and rice, a number of studies have suggested that homologs of *CO* fulfill comparable roles in other species. These include the dicots *Brassica nigra* L. (Robert et al., 1998), Japanese morning glory (*Pharbitis nil* (L.) Choisy) (Liu et al., 2001), and potato (*Solanum tuberosum* L.) (Martínez-García et al., 2002; Beketova et al., 2006) as well as monocots such as rice (Yano et al., 2000), common wheat (Nemoto et al., 2003), perennial ryegrass (*Lolium perenne* L.) (Armstead et al., 2005; Martin et al., 2005), and barley (*Hordeum vulgare* L.) (Griffiths et al., 2003). In some, although the behavior of *CO* itself appears to be the same,

completely different photoperiod-dependent processes are being regulated. In potato, tuber development and other associated morphological changes rather than flowering (Martínez-García et al., 2002; Rodriguez-Falcon et al., 2006). Although modern potato cultivars have been selected for relative insensitivity to day length, some wild species such as *Solanum demissum* and *S. tuberosum* ssp. *andigena* are strictly dependent on short days for tuber formation (Ewing and Struik, 1992). whereas in aspen (*Populus trichocarpa*) *CO* regulates both flowering and the processes of bud set and growth cessation in response to shortening days in the fall (Böhlenius et al., 2006). Expressed homologs of *CO* are also known in species with no known photoperiodicity, such as apple (for example, GenBank accession EB141172 and others) and tomato (Drobyazina and Khavkin, 2006), though their function, if any, remains unclear.

Not only is *CO* well conserved among species, but so are most of the other components of the system regulating flowering, both upstream and downstream. Even rice, a short-day monocot, contains homologs of nearly all the major components described in Arabidopsis (Izawa et al. 2003). Similar pictures are emerging in poplar (Böhlenius et al., 2006; Hsu et al., 2006; Mohamed, 2006), potato (Rodriguez-Falcon et al., 2006), and apple (Wada et al., 2002; Kotada and Wada, 2005; Kotada et al., 2006) and this seems likely to be the case in most plant species.

Although the molecular basis for the variation in flowering habit seen in *Fragaria* is still unknown, such variations in other crops have been shown to related directly to alterations in this small group of critical genes. Researchers in Japan have demonstrated that two major QTL affecting heading date, *Hd1* and *Hd3a,* correspond to orthologs of *CO* and *FT*, respectively (Yano et al., 2000; Kojima et al. 2002), whereas a third heading-date QTL, *Hd9*, has been found to be tightly linked to the rice homolog of *SOC1* (Tadege et al., 2003). Photoperiod insensitivity in barley has been shown to result from disruptions in the circadian oscillation of *CO* caused by

mutations in *Ppd-H1*, a member of the pseudo-response regulator class of genes involved in circadian clock function (Turner et al., 2005)

Using Model Systems To Understand Development In Rosaceae

Research to date has provided a detailed understanding of the gross physiology of flowering and development in fruit crops such as strawberry, while work in models such as Arabidopsis and rice have provided most of what is known about the mechanisms at work at a molecular level. To truly capitalize on these bodies of work it is necessary to find ways of integrating information from both.

This is already beginning to occur in apple. Studies have identified and characterized apple orthologs of many of the components known in Arabidopsis, including *AFL1* and *AFL2* (apparent equivalents of *LFY*), *MdAP1, MdTFL1*, *MdFT*, and over a dozen MADS-Box transcription factors involved in reproductive development (Sung et al., 1999; Yao et al., 1999; Kotoda et al, 2000, 2002; Wada et al., 2002; Kotoda and Wada, 2005). In general, such research has shown the components in apple to play roles similar to the homologous Arabidopsis genes. *MdAP1* and the pair of *LEAFY* homologs, *MdLFY-1* and *MdLFY-2,* have been found to promote flowering in transgenic Arabidopsis, and appear to be involved in the promotion of flowering in apple as well (Kotoda et al., 2000; Wada et al., 2002). Similarly, *MdTFL1* has been shown to retard the transition to flowering in transgenic Arabidopsis (Kotoda and Wada, 2005) and suppression of *MdTFL1* through expression of antisense construct of the gene has been demonstrated to shorten the vegetative juvenile phase of 'Orin' apple trees from more than six years to as few as eight months (Kotoda et al., 2006). Some studies, however, have shown that mechanisms in apple behave differently than would be predicted by the Arabidopsis model. For example, overexpression of *AFL1* in apple, unlike overexpression of the similar *LFY* in Arabidopsis, does not result in precocious flowering (N. Kotoda, unpublished research cited in

Kotoda et al., 2006). Regulation of *LFY* in Arabidopsis is primarily controlled by photoperiod (Samach, 2000) and apple, an apparently day-neutral species, may regulate flowering through a pathway not primarily dependent on *LFY* orthologs.

Classical physiology has already offered numerous hints of what may be occurring in strawberry at the molecular level. Several researchers (Vince-Prue and Guttridge, 1973; Thompson and Guttridge, 1960) have suggested that the evidence supports the existence of a phloem-mobile inhibitor of flowering, synthesized in the leaves. This is reminiscent of the inhibitory effects of *CO* in rice (Yano et al., 2002), on *FT*, which along with *TFL1,* has been shown to move from the leaves to the meristem in Arabidopsis (An et al., 2004). Similarly, the effects of light quality on flowering as shown by Vince-Prue and Guttridge (1973) suggest the possibility of phytochrome-mediated regulation of a hypothetical *CO* homolog. To truly understand what is occurring within the plant in each of these cases, however, will require the development of the needed molecular tools.

The well-documented complex network of factors affecting flowering in strawberry: photoperiod, light quality, temperature, vernalization, all have corresponding mechanisms documented in the molecular models. These mechanisms may not prove identical in strawberry, and in some cases may prove radically different, yet this knowledge can act as a foundation for investigations into the interactions between genes and environment that control vital aspects of the crop's development.

To date little such work has been conducted in strawberry. Yet in many ways strawberry represents an attractive target for such studies—it has clearly delineated flowering phenotypes, a short life cycle, ease of hybridization, and convenient plant size. The octoploid genome number and alloploid nature of the cultivated species represent challenges, but the many diploid relatives

have much simpler and smaller genomes. Like apple, strawberry is an important crop species, and a better understanding of the genes critical to its development might have economic impacts. Such knowledge might lead to improved cultivars and more efficient cultural practices, benefiting the farmer, consumer, and environment.

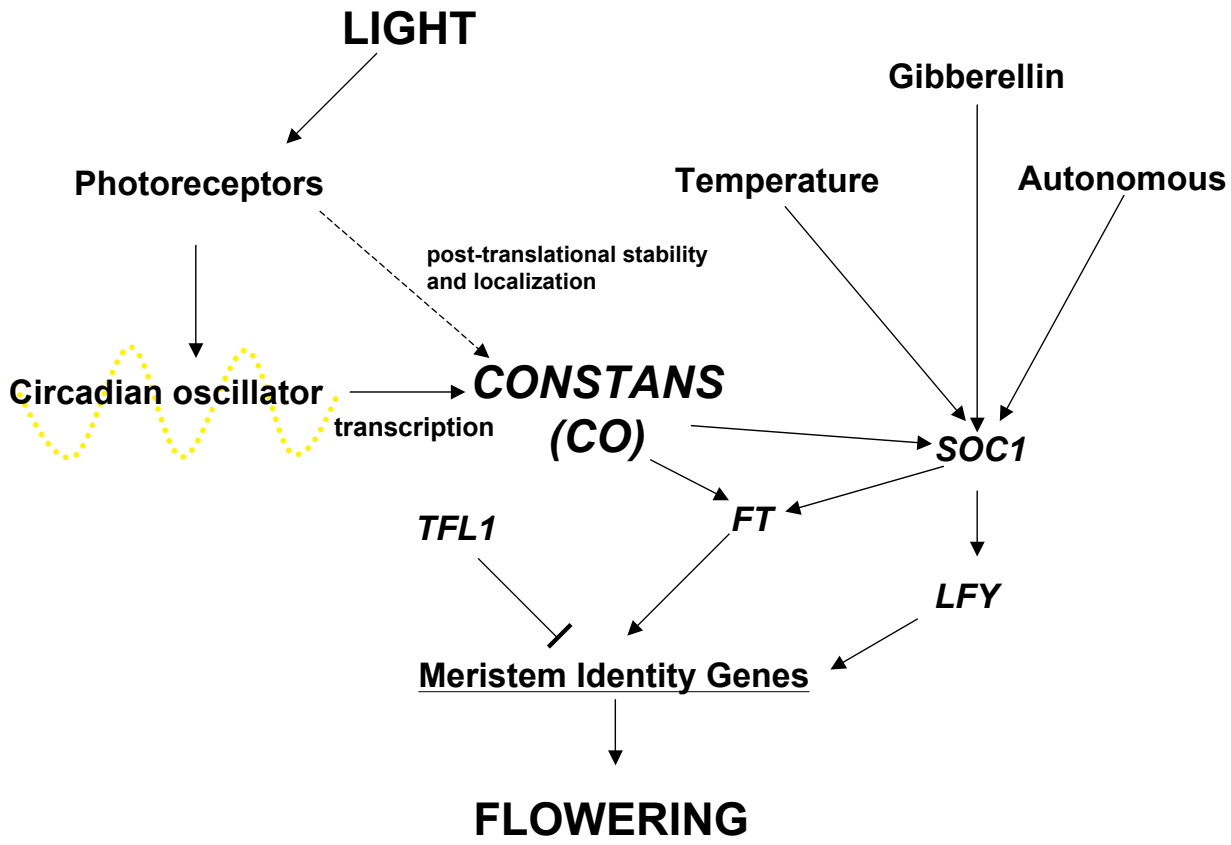

Figure 2-1. Simplified diagram showing interactions of genes and environmental factors governing flowering in *Arabidopsis thaliana* (arrows represent promotive effects, bars represent repressive effects).

CHAPTER 3
CHARACTERIZATION OF CONSTANS-LIKE GENES IN *Fragaria*

Introduction

The initiation of flowering is a critical developmental milestone in most plant species and represents a vital process for both natural evolution as well as crop production and development. The mechanisms involved in switching between vegetative and reproductive development have been shown in model species to be precisely regulated by a number of pathways, governed by both internal and environmental factors. Among these is photoperiod, which is critical to the growth and development of many crop species, including the cultivated strawberry. As in many other species, the timing of flowering in strawberry is an important factor with major impacts not only on the adaptation of cultivars to particular locales or cultural methods, but also, because of the relationship with the timing of the subsequent harvest, plays a vital role in determining the market value of the crop as well.

Despite a narrow genetic base (Sjulin & Dale, 1987), commercial strawberries display a wide spectrum of sensitivities to photoperiod, ranging in phenotype from strictly short-day (SD) and "long-day" everbearing (EB) cultivars to day-neutral (DN) types that flower regardless of photoperiod. Because these different types share overwhelmingly similar genetic backgrounds and may even co-exist in the progeny from a single cross (Ahmadi et al., 1991), the differences are likely attributable to the qualitative and quantitative attributes of a small number of critical genes, thus strawberry provides a unique model system to investigate this important pathway. The ability to characterize the molecular basis for these differing phenotypes could have important implications for cultural techniques and cultivar development not only in *Fragaria*, but also potentially in the other cultivated members of the Rosaceae, for which timing of flowering is also a critical factor.

Studies in strawberry allow direct translation of molecular paradigms devised in long-day and short-day model systems to a single species with ranging photoperiodic flowering habits. Studies in Arabidopsis have shown that the gene *CONSTANS* (*CO)* plays a critical central role in the regulatory pathway responsible for photoperiodic flowering (Simpson and Dean, 2002; Corbesier and Coupland, 2005), acting as a dynamic link between photoreceptors, the circadian oscillator, and the genes governing meristem-identity. Mutations in the *CO* gene cause delayed flowering in Arabidopsis, a LD plant (Putterill et al., 1995), and early flowering in rice, a SD plant (Yano et al. 2000). Its function as a regulator of flowering is well-conserved across species, having been characterized in both other eudicots (Robert et al., 1998; Liu et al., 2001) and monocots (Yano et al., 2000; Martin et al., 2004; Nemoto et al., 2003), though the downstream effect varies between species, either suppressing or promoting flowering. *CO* is one member of a large family of *CONSTANS*-like genes, numbering 17 in Arabidopsis, 16 in rice (*Oryza sativa*) , and at least nine in barley (*Hordeum vulgare*) (Griffiths et al. 2003). No accounting of these genes appears to have been previously published in strawberry.

The high degree of conservation in both structure and function, even between monocots and eudicots, suggests that *COL* genes likely have important roles in controlling flowering time across species. A similar situation is likely the case within *Fragaria*, yet its diverse flowering habits suggest that regulation may be imparted through novel alleles of known genes, uncharacterized patterns of expression, or pathways that circumvent photoperiodic influence by acting on elements downstream from the photoperiodic regulators. This study proposes to identify, catalog and characterize probable *COL* genes in *Fragaria*, describe tissue specific, temporal and photophysiological expression patterns. The results of this study provide additional

information about into the mechanisms that regulate photoperiodic development. These results have implications for the evolution of the gene family in Rosaceae.

Nomenclature

Because of the difficulties in coordinating the numbering of family members across species that may possess different numbers of such genes, a decision was made to simply number the *COL* genes in the order they were found, rather than attempting to match numbering of Arabidopsis genes. In this report, *FrCOL* is used to refer to genes at a particular locus across members of the genus *Fragaria*, whereas *FraCOL, FrvCOL, FrnCOL,* and *FriCOL* are used to designate genes of *F.* ×*ananassa*, *F. vesca*, *F. nubicola*, and *F. iinumae*, respectively. Aside from numbering, genes were named for their apparent Arabidopsis homologs.

Results

Cloning And Identification Of Four CONSTANS-LIKE Genes Of *Fragaria*

A series of EST libraries have been generated by this laboratory, representing transcripts accumulating in mature *F.* ×*ananassa* plants (Folta et al., 2005), developing flower buds, various root tissues, 12 fruit stages (K. Folta et al., unpublished) and *F. vesca* seedlings (J. Slovin et al., unpublished). These EST collections served as a basis for gene discovery based on direct sequencing, colony hybridization and PCR with degenerate primers.

Simple homology comparisons to sequences within public databases identified convincing homologs of at least five distinct *Constans*-like (*COL*) genes. Four were designated *FrCOL1*, *FrCOL2*, *FrCOL3,* and *FrCOL4*, whereas the fifth, which shared the greatest homology with *AtCO* and the rice *Hd1*, was presumed to be the *Fragaria* ortholog of *CO* and designated *FrCO*. These genes represent the canonical three distinct subgroups of the *COL* family as outlined by Griffiths et al. (2003) (Figure 3-1). Close matches to each of these genes were found among publicly available ESTs in other Rosaceae species as well (Table 3-1).

A full-length cDNA clone of *FraCO* was obtained from a flower tissue library derived from *F.* ×*ananassa* 'Strawberry Festival'. Seven individual clones were identified and sequenced, and all contained transcripts completely identical except that two contained a somewhat truncated version of the 3' UTR. The 1,197 bp coding region shows considerable similarity to *AtCO* and other previously characterized homologs, with the most similar known transcript being *PdCO1*, from *Populus* (Table 3-1). Comparison of the amino acid sequence to those proteins designated as Group I suggested that *FrCO* belongs in Group Ia (Figure 3-2), a division that includes all genes experimentally demonstrated to complement the Arabidopsis *co* mutant and those believed most likely to play an important role in flowering behavior. The predicted protein contains a CCT domain and two intact B-Box regions, more closely resembling similar proteins in other eudicots, rather than the less conserved second B-Box present in some monocots (Martin et al., 2004). All four middle domains common to Group Ia COL proteins were also identified.

Full-length coding sequences for three other *FrCOL* genes identified could be deduced from overlapping *F. vesca* ESTs, as well as part of a fourth. Although these genes contained the basic structures of *COL* genes, they bore less similarity to *AtCO* than did *FrCO* and appear unlikely to be the *Fragaria* ortholog of *CO*. These were designated *FrCOL1*, *FrCOL2*, *FrCOL3,* and *FrCOL4*.

FrCOL1 and *FrCOL2* both encode predicted proteins consistent with Group Ic *COL* genes, with the major difference from the Group Ia genes being the presence of the M2c (Zobell et al., 2005) rather than the M2 domain (Figure 3-3). Several copies of both were identified among *F. vesca* EST sequences, as well as a single, slightly different, *F.* ×*ananassa* 'Strawberry Festival' *FrCOL1* sequence. FrCOL1 is roughly equal in similarity to both *AtCOL3* and *AtCOL4*

at the amino acid level (55%), with *AtCOL3* having slightly higher similarity in the major functional domains. It is most similar to *MdCOL1* and *MdCOL2* from apple, the only previously characterized *COL* genes in the Rosaceae (Jeong et al. 1999), and to *PrpCO*, a *COL* gene from peach mapped by Silva et al. (2005). *FrCOL2*, while retaining a similar structure at the amino acid level, was not closely related to *FrCOL1* at the nucleotide level, suggesting the two genes diverged some time ago. Among Arabidopsis *COL* genes, the predicted protein most closely resembled that of *AtCOL5*. Based on their relative frequency in BLAST-X searches of Rosaceae ESTs, *FrCOL1* and its orthologs are possibly among the most commonly expressed *COL* genes in the family, whereas *FrCOL2* and its homologs are expressed at a somewhat lesser level (data not shown).

FrCOL3 was the least *CO*-like of the transcripts identified, with a structure consistent with a classification in Group II. *FrCOL3* was found only in *F. vesca* libraries derived from young seedlings, and although matches were found among Rosaceae ESTs (primarily in an apple seedling library), it appears to be a much rarer transcript than the *FrCOL1* and *FrCOL2* genes.

Only a partial sequence for *FrvCOL4* was found among the sequenced ESTs, comprised of the B-Box region and much of the 5' UTR. Although the full sequence was not available, this clearly represents a gene distinct from those previously described. The closest match in Arabidopsis was *AtCOL10*, indicating this is a likely member of Group III, and if so, all four groups present in Arabidopsis have also been identified in *Fragaria* (Figure 3-2). No members of Ib, Id, Ie, or IV were found, none of which have thus far been identified in eudicots (Griffiths et al., 2003).

FrCO/*FrCOL* Gene Structure

Group I and II *COL* genes in Arabidopsis possess a single intron located about a third of the length from the 3' end of the coding region, between the M3 and M4 regions (Fig. 3-4).

Using internal primers designed to include ~400 bp of the coding region and to flank the putative intron site (Table 3-2), based on the previously identified cDNA, partial genomic versions of *FrCO, FrCOL1,* and *FrCOL2* were amplified and sequenced from genomic DNA of *F.* ×*ananassa* 'Strawberry Festival', *F. vesca* FDP815, and *F. nubicola* FDP601 (Figure 5-4.) Additionally, a product was also amplified in *F.* ×*ananassa* flower cDNA, using the same primer sets.

In all cases, an intron was shown to be present at the predicted site in the amplified genomic product (Figure 3-4). *FrCO* intron length ranged from 704 to 746 bp. Introns in *F. nubicola, F. vesca,* and three nearly identical alleles from *F.* ×*ananassa* (*FaCO*-35) were highly similar in both sequence and length (704, 707, and 706 bp, respectively), whereas a second *F.* ×*ananassa* allele (*FraCO-C9*) was considerably different, containing a 28 bp insertion and numerous other polymorphisms, for a total length of 746 bp. Somewhat similar to this allele were those of *F. iinumae* and *F. nubicola* (Figure 3-5). Alleles also differed significantly in sequence at one site in the coding region, between the M1 and M2 domains. This region appears to be characterized by highly repetitive sequence containing multiple CAA codons, and is poorly conserved between and even within species (Figure 3-5A,B). *FraCO-C9* and *F. iinumae* were considerably divergent in this region from the other alleles sequenced (Figure 3-5). *FrCOL1* was shown to contain a 82 bp intron in both *F. x ananassa* and *F. vesca*, similar in length to the related *AtCOL3* (101 bp), but considerably smaller than that of *AtCOL4* (403 bp). Additionally, a 12-bp insertion was identified in a single 'Strawberry Festival' EST, designated 7C. A primer landing in this insertion amplified a product in all eight octoploid cultivars tested, but not in any of the diploids, suggesting that this particular insertion may be unique to the octoploids.

FrvCOL2 contained a 118 bp intron, whereas *FrnCOL2* a 119 bp intron, both similar in length to the 88 bp intron of AtCOL5. The cDNA product for *FrCOL1* was the same size as that predicted from the sequenced transcript. However two product sizes were observed for *FrCOL2*, consistent with the presence of an unspliced transcript variant, and a partial EST transcript containing nearly all of the intron sequence was identified among *F. vesca* sequence data.

Southern blot analysis of *FrCO* Copy Number

Southern blot analysis displayed a banding pattern consistent with a single *FrCO* locus in *F. vesca*, as in other diploid organisms, showing two bands in those lanes where the probe fragment was cut once by the chosen enzyme, and one in those where it was not cut. The cultivated octoploid, *F. ×ananassa*, however, showed multiple bands, frequently as many as six for enzymes that cut with in the probe region (Figure 3-7).

Mapping Of *Fragaria CO* And *COL* Genes In A Diploid Mapping Population

The fragment of *FrnCO* generated by the RN-FaCO primers was found to lack one of the two *Hind*III enzyme restriction sites possessed by the corresponding fragment of *FrvCO*, and this was used to generate a CAPS marker for mapping (Figures 3-8A, 3-8B). This marker segregated in a 14:24:18 (aa:ab:bb) ratio in a subset of the reference population developed by Sargent et al. (2004), and was found to map to linkage group VI, with linkages to SSR markers EMFn153, UDF025, FAC-005, and EMFv160AD, with LOD values of 15.48, 7.79, 11.15, and 13.71 respectively (Figure 3-8C).

No polymorphisms were found between *F. vesca* FDP815 and *F. nubicola* FDP601 in the intron and flanking coding region of *FrCOL1*. Two polymorphic restriction sites were identified in *FrCOL2*, and mapping was attempted first using EcoRV. Because of suspect segregation ratios and banding patterns that were difficult to resolve, a decision was made to score the marker as a dominant marker, but the incongruous segregation ratio persisted, yielding a 40:41

(aa:b_) ratio. The first 24 individuals were also scored using the second enzyme, *Afl*III, yielding the same pattern seen among these plants with the first enzyme. The resulting mapping data did not convincingly place the gene on any of the seven linkage groups or in a strong linkage relationship with any of the established markers.

Comparison Of Strawberry Genotypes Under Varying Photoperiods

In order to test the expression of *FrCO* and other genes under various photoperiods, a pair of growth chamber experiments were performed. The first growth chamber experiment consisted of a comparison of the SD cultivar Strawberry Festival with the DN cultivar Diamante under 8 and 16 h photoperiods. After several weeks under these treatments, these genotypes reacted as predicted, with 'Strawberry Festival' flowering heavily under SD and producing numerous runners under LD, and 'Diamante' flowering vigorously under both photoperiods. The DN plants produced runners only under the 16 h photoperiod, and even then only produced a very few runners. Although observations were made, no flower or runner data were collected in this experiment.

The second growth chamber experiment, under cooler temperatures, compared 'Diamante' to the SD cultivar Camarosa, and to a selection of *F. vesca*, Hawaii-4. 'Diamante' again flowered under both conditions, more vigorously under LD than SD. 'Camarosa' did not runner and flowered only under SD. Unexpectedly, Hawaii-4, a yellow-fruited diploid selection presumed to be closely related to *F. vesca* ssp. *semperflorens* and previously classified as not photoperiod-sensitive (Oosumi et al., 2006), behaved in a clearly SD fashion, flowering profusely under an 8 h photoperiod and runnering primarily under LD (Table 3-4).

The expression pattern of *FraCO* in the first study was studied by RNA-gel (northern) blot and subsequent hybridization to a radio-labeled CO probe. Transcript levels varied both between plants grown under SD and LD conditions, as well as between SD and DN genotypes

(Figure 3-9). Under LD, transcript level in 'Strawberry Festival' peaks rather weakly before dawn, whereas under SD a strong peak appears in the early part of the day. Transcript levels in 'Diamante', however, are lower under both conditions, with a broader peak in the afternoon, later than in 'Strawberry Festival', and almost no transcript present under long days.

One concern was that RNA-gel blot experiments may not give precise measurement of CO transcripts because of potential cross-hybridization with other family members. To verify the results, gene-specific primers were developed and used to evaluate steady-state RNA levels in a similar experiment, this time using semi-quantitative RT-PCR. Similar results were seen when the growth chamber experiment was repeated with 'Diamante', 'Camarosa', and Hawaii-4 (Figure 3-10) using these methods. All cDNA samples were tested with primers for ubiquitin and the ubiquitin control was used to balance template amount across samples within each set.

Allele-Specific Expression Patterns Differ Betwen Short-Day And Day-Neutral Genotypes

Although allele-specific primers for the *FraCO* allele types 35 and C9 amplified products from genomic DNA of 'Strawberry Festival' and 'Diamante', 33 cycles of RT-PCR showed that while both alleles are actively expressed in 'Diamante', only the *FraCO-35* allele appears to be significantly expressed in 'Festival' at dawn under short days, although a trace of *FraCO-C9* is visible. A more visible band appeared at 40 cycles, suggesting that trace amounts may still be expressed, although this may be the effect of buildup from slightly non-specific priming (Figure 3-11). Additionally, though the amount of *FraCO-35* and *FraCO-C9* product present at 33 cycles in 'Diamante' seems similar, the amount of *FraCO-35* product present in 'Strawberry Festival' appears higher than either allele in 'Diamante'. No differences were noted in the expression of the 7C allele of *FraCOL1*, which appeared to be actively and equally expressed in both cultivars (not shown). As before, template levels were adjusted by equalizing with ubiquitin controls.

Expression Of Other Genes Under Different Photoperiods

Expression of other *COL* genes in these samples was also been investigated using RNA from the preceding experiments to see if their expression correlated in some way with relevant phenomena. From the first experiment, cDNA was generated from RNA taken at the 5 AM time point (the point of peak *FraCO* expression under SD conditions, corresponding with hour 8 in Figure 3-9) and from the low point of *FraCO* expression, 5 PM, in the SD and LD 'Strawberry Festival' and 'Diamante' sample sets. These cDNA samples were used as template for RT- PCR to compare quantitative differences in expression of the *CO* and *COL* genes, as well ubiquitin and actin as controls (Figure 3-12). The previously noted difference in *FraCO* level was observed again, and was accompanied by higher levels of *FraCOL2* under short days, but *FraCOL1* appears more highly expressed under long day conditions. No measurable levels of *FraCOL3* transcript were observed under either condition in either cultivar. All transcripts except *FraCOL1* were undetectable at the 5 pm time point under both SD and LD conditions, coinciding with the low point in *FraCO* expression seen in the previous experiment, suggesting that they too may cycle diurnally. Control genes were uniform between treatments, suggesting that the differences observed in specific targets were reflective of actual variations in transcript level.

The same process was repeated for the entire diurnal time course of *F. vesca* Hawaii-4 from the second photoperiod experiment. In this case, very little expression of *FrvCOL1* and *FrvCOL3* were seen. *FrCOL2* showed some evidence of circadian cycling, but not the large difference in amplitude between SD and LD seen with *FrCO*, and in fact seemed to show evidence of a 12 hour period of variation (Figure 3-13).

Some *FraCOL2* Transcripts Contain An Unspliced Intron

Under some conditions, a second product of higher molecular weight was visible for *FraCOL2*. The predicted size of this fragment was consistent with the retention of the 118-119 bp intron shown in the diploid *FrCOL2* genes. Additionally, a partial *FrvCOL2* EST, DY674801, was identified in Genbank with more than 95% of the intron, the sequence ending only a few bases short of the end. Conditions under which the unspliced transcript was detected varied between genotypes. In 'Strawberry Festival', it was present only under LD conditions, at the 5 am time point, whereas in 'Diamante' it was visible only under SD conditions, but at both 5 am and 5 pm (not shown). In *F. vesca*, in the second trial, however, it was clearly visible at two points of the day under short photoperiods (Figure 3-12).

Discussion

The *Co* Gene Family In *Fragaria* (Rosaceae)

The results of these studies clearly demonstrate the presence of a family of *COL* proteins in *Fragaria* similar to those previously demonstrated in a number of other species, accounting for all subgroups demonstrated in Arabidopsis and including a likely homolog of *CO*.

The five members of the *COL* family identified in this work cover the full range of *COL* diversity described in the Arabidopsis, the only eudicot in which the entire family is known to be documented, with representatives in all subgroups. Unlike in Arabidopsis, only a single member of Ia, *FrCO*, was identified. Unless there is significant deviation at the nucleotide level, the possibility of other Ia *COL* genes in *Fragaria* seems remote. Both the results of the *F. vesca* Southern blot, and the fact that no others were isolated in two hybridization experiments, one probed with *AtCO* and the other with *FraCO*, suggest that other such genes may not exist in strawberry. No evidence of multiple group Ia loci was seen among ESTs from other diploid Rosaceae species, although two distinct but closely-related classes of *CO* were found in apple.

Multiple group Ia genes are common, even in diploid species, however, and have been demonstrated in poplar, tomato (*Lycopersicon esculentum* Mill.), barley, and oilseed rape (*Brassica napus* L.) as well as in Arabidopsis (Fig. 3-1), but have been shown not to be the case in rice (Yuceer et al., 2002; Ben-Naim et al. 2006; Griffiths et al., 2003; Robert et al., 1998). It is possible that rather than possessing distinct roles, the non-*CO* group Ia genes in these species are merely the result of duplication, coupled perhaps with at least partial loss of function within the genome, although *AtCOL1* has been shown to shorten the circadian cycle when overexpressed in wild type plants (Ledger et al. 2001). *AtCO* and *AtCOL1* are very similar, even at the nucleotide level, but *AtCO* overexpressors show no change in circadian cycle. However it is possible that reciprocal mutations have separated functions of the original gene into the two genes, with the current *AtCO* retaining the ability regulate flowering and *AtCOL1* possessing a role in circadian regulation.

Although *FrCO* mapped to the same linkage group in the diploid population as the seasonal flowering locus (*SFL*), there was no significant linkage with this locus, as 38cM separate the two, casting doubt on the idea that non-seasonal *F. vesca* may be *FrCO* mutants. Neither of the two QTLs for date of first flower in the diploid mapping population mapped to the location of *FrCO* (Sargent et al., 2006a). This suggests that variation in *FrCO* is not a significant source of variation in flowering time in this population, and that unlike in other species, such as rice (Yano et al., 2002), the lack of seasonality is not the result of mutations in the *CO* gene.

Homologs of all *Fragaria COL* genes were found in ESTs from other Rosaceae species, though in no case were homologs of all five genes found in a single species (Table 3-3). This is most likely due to the limited number ESTs available from a given species, as *COL* proteins are

generally relatively low in abundance and only apple begins to approach the large numbers of ESTs need to reliably locate rare transcripts.

Searches of publicly availabe ESTs identified group Ia genes in both *Malus* and *Prunus* species, and it appears that there may be two distinct members of the group in apple. Unlike strawberry, apple does not appear to be photoperiodic (Carew and Battey, 2005). However, group Ia *COL* genes nonetheless appear to be present in all higher plant species thus far investigated, including some that lack photoperiodic sensitivity, such as tomato (*Lycopersicon esculentum*) (Ben-Naim et al. 2006).

Group Ic, homologs of *AtCOL3/4/5* represented in *Fragaria* by *FrCOL1* and *FrCOL2*, appear to be among the most highly expressed *COL* genes in the Rosaceae, constituting 6 of 19 *COL* ESTs identified in *Fragaria*, 13 of 20 in *Prunus*, and 87 of 106 in *Malus,* of which a large majority are homologs of *FrCOL1*. Prior to this study, the only Rosaceous genes yet characterized were members of this group, a single pair from *Malus domestica*, *MdCOL1* and *MdCOL2* (Jeong et al, 1999). A very similar gene designated *PrpCO* was also mapped to *Prunus* linkage group G1 on *P. dulcis* × *P. persica* F_2 map by Silva et al. (2005), but was not further characterized.

The group Ic genes may be the most ancient members of the family, being the only *COL* genes identified in the primitive plant *P. patens* (Zobell et al. 2005). As with all non-*CO* members of the family, the function of these genes is still unclear. Zobell (2006) found no discernible phenotype in single mutant knockouts for any of the three members in *P. patens*. However, work by Datta et al. (2006) has shown early flowering and changes in lateral branching and root development in Arabidopsis *COL3* knockout mutants, and have suggested a

role as a positive regulator of red light signaling. *FrCOL1* and its homologs may have similar functions in the Rosaceae.

Although two polymorphisms suitable for mapping were detected, mapping *FrCOL2* in the diploid reference population proved difficult, and it was not possible to identify linkages with any published markers. Scored as a dominant marker, one would anticipate a segregation ratio of 3:1, but in this case the pattern was close to 1:1, skewing towards the *F. vesca* parent. Some parts of this map have shown considerable distortion (Sargent et al., 2004a, 2006b), including one marker, BFACT-010, that segregated 79:5 rather than 3:1 (Sargent et al., 2006b). This distortion of segregation ratios may be due to the parents chosen as parents of the reference population. Although Sargent et al. (2004a) produced their population from two selfed F_1 *F. vesca* x *F. nubicola* hybrids, previous researchers found F_1 plants to be self-incompatible, like the *F. nubicola* parent (Evans and Jones 1967) or sterile (Dowrick and Williams, 1959). Although the mechanics of self-incompatibility have yet to be elucidated in strawberry, it may be that this system remains functional to an extent, resulting in bias in what pollen may successfully fertilize the F_1 plants. Additionally, although sexually compatible, *F. vesca* and *F. nubicola* do not overlap in their ranges and are distinct on both a morphological and molecular level (Sargent et al. 2005, 2006b; Potter et al., 2000) suggesting that there has been time for divergence between these species. Such divergence might result in imperfect pairing of chromosomes, and if large-scale rearrangements exist between the species, may even be reflected in the formation of multivalents and gametes with chromosomal abnormalities.

FrCOL3 is the sole representative of group II *COL* genes identified. While maintaining the general *COL* structure, group II genes have only one B-Box and the four conserved middle regions. Though common to both monocots and dicots, comprising 4 of the 17 Arabidopsis *COL*

genes and 3 of the 16 in rice, their function remains unknown, and this may represent the first report of such a gene outside of these two model systems. Apparent homologs of *FrCOL3* were identified among ESTs of only one other Rosaceae species, peach, although a distinct transcript matching the criteria of a group II *COL* gene was found among apple ESTs as well.

Mining of EST libraries revealed only one partial transcript of *FrvCOL4*, consisting of the 5' end the gene. Although we can only guess at the rest of the gene's structure, this end is clearly characteristic of a class of *COL* gene not otherwise represented by the other *FrCOL* genes, group III. The B-Box region of group III genes is distinctive because instead of a pair of B-Boxes, these genes have a single B-Box followed by a modified zinc-finger region. Although impossible to observe in an EST, these genes are also distinguished from the others in the family because of their differing intron structure, with two splice locations in the middle of the gene and a third within the CCT region, none of which correspond to the intron location in the other family members (Griffiths et al. 2003). This suggests that the differentiation from the other *COL* genes is very ancient.

The function of this class of genes is still imperfectly known, but *AtCOL9* has been shown to play a role in flowering, apparently through the suppression of *CO*, and, as a result, *FT* (Cheng & Wang, 2005). A related gene, *AtCOL10*, was also shown to have similar role in early work presented by the same authors (Cheng & Wang, 2006), and both appear to be expressed in a diurnal fashion.

Evolutionary Relationships Among *FrCO* Sequences

The relationships between the octoploid *FrCO* alleles and those of the diploid species reinforce some aspects of the established views of the origins of the diploid genomes of *F.* ×*ananassa*, while perhaps casting doubt on others.

One allele class, *FraCO-35,* bears striking resemblance to that of *F. vesca*. Within the coding region, only the previously mentioned variable site between the M1 and M2 regions differs, and in fact this region differs considerably even between two selections of *F. vesca*, Hawaii-4 and FDP815. Even within the intron, the nucleotide sequence is 98% identical between *FraCO-35* and *F. vesca* (Figure 3-5).

The idea that *F. vesca* may represent a diploid progenitor of the octoploids is an old one, suggested first by Ichijima (1926) and reinforced by further work, both cytogenetic (Senanayake and Bringhurst, 1967) and molecular (Potter et al, 2000; Folta and Davis, 2006; also Chapter 5 of this dissertation) and this result seems to strengthen this view.

The origin of the other component genomes has never been as clear, though a number of species have been suggested. The sequence of the other type of octoploid allele, *FraCO-C9*, does not seem to clearly implicate any of these, but may indirectly point to two species, *F. nubicola* and *F. iinumae*. *F. nubicola* has long been discussed as a progenitor of the octoploid species, and was suggested as a possible source of the A or A′ genomes by both Senanayake and Bringhurst (1967) and Potter et al. (2000). Staudt (1989) had proposed *F. nubicola*, along with *F. vesca*, as the ancestors of the hexaploid species *F. moschata*, but sequence data analyzed by Potter et al. (2000) implicates *F. orientalis*, and Lin and Davis (2000) showed that *F. viridis* was the likely cytoplasm donor for *F. moschata*. The range of *F. nubicola,* today confined to a relatively small area of South Asia, does not overlap the ranges of *F. moschata, F. viridis*, or *F. vesca.* If *F. nubicola* is in fact an ancestor of both *F. moschata* and the octoploid species, this may suggest that it or a closely related species once existed across a much broader geographic area.

F. iinumae has primarily been implicated in unpublished work by DiMeglio and Davis (cited in Folta and Davis, 2006). Currently, populations of *F. iinumae* are confined primarily to the island of Hokkaido and the Kuril Islands. The species appears to be an outlier from the rest of the genus both at the morphological level (Sargent et al., 2004b), and at the molecular level (Harrison et al, 1997; Potter et al., 2000, Sargent, 2005). *F. iinumae* has proven incapable of producing hybrids with other diploid species (Bors, 2000; Sargent et al., 2004b) but polyploids derived from the species do not appear to have been investigated and may in fact be capable of producing allopolyploid offspring in combination with other species.

No diploid species contained the 28bp insertion in the intron of the *FraCO-C9* allele, and *F. iinumae* contained 5bp and 7bp intron deletions not seen in *FraCO*-C9 or any other allele. However, an inspection of SNPs within the intron on either side of the 28 bp insertion reveals an interesting pattern—on the upstream side of the insertion, C9 and *F. nubicola* share the same base at 7 of 8 SNP sites, compared to only 1 between C9 and *F. iinumae*. Downstream of this insertion, however, more SNPs match *F. iinuame*, with 9 of 18, compared to 6 matches between C9 and *nubicola* and 3 sites where C9 did not match either species. Small microsatellite repeats in the intron, all downstream of the insertion, also seem to more closely resemble *F. iinumae*, with two of the three identical between the two alleles (though the difference between *F. nubicola* and the other two alleles is only one repeat in both cases), and the third SSR containing five repeats of AT in *FraCO-C9*, four in *F. iinumae*, and three in all other sequenced alleles. The coding regions of the gene are nearly identical among all alleles, with the exception of the variable region between the M1 and M2 domains, which matched exactly between *FraCO-C9* and *F. nubicola*. Given the level of polymorphism in this region, with even members of the same

species showing variability, this evidence would seem to make a strong case that at least this portion of the gene originated in *F. nubicola*.

The division seemingly indicated by the 28 bp insertion may represent a slightly uneven crossing over event in the species' past, creating a chimeric gene with an upstream end derived from *F. nubicola* or a near relative, and a downstream end derived from *F. iinumae* or an ancient related species. If this locus lies in a region characterized by frequent crossover or disruption by interchanges between genomes, this may explain the failure to express of the *FraCO-C9* allele seen in 'Strawberry Festival', for example because of damage to the promoter. Nemoto et al. (2003) found that the *CO* locus in one of the three genome pairs of hexaploid was not expressed, despite an intact coding region, and traced this to an 63 bp deletion in the promoter upstream from the gene.

It seems unlikely that these two classes represent the entire octoploid complement of *FraCO* allele types. Only *FraCO-35*-type sequences were identified in sequencing of cDNA from 'Strawberry Festival', and only two identical *FraCO-C9* sequences were identified (by the size difference caused by the insertion and by failure to cut with *Xba*I) among 16 clones amplified from genomic DNA of 'Strawberry Festival'. It is possible that alleles are not being identified by the PCR-based methods used in this study due to mismatches in primer sequence, or that the *FrCO* locus has been eliminated from one or more of the component genomes of the octoploid.

FrCO **Expression**

In previously characterized SD plants, such as rice (Yano et al., 2000) and perennial ryegrass (Martin et al., 2004), *CO* expression patterns remained about the same as that seen in Arabidopsis. The differences in flowering behavior come as a result not of differences in *CO*

expression pattern, but from differences in the output, with *CO* acting as an inhibitor of *FT* and *SOC1,* and hence flowering, rather than promoting them as in Arabidopsis.

In strawberry, it appears that something quite different is occurring. Under short days, *FrCO* transcript levels conform to a robust diurnal cycle, though the peak is shifted so that unlike under SD in Arabidopsis, it occurs during the day, rather than after dusk. Under long days, however, there is little *FrCO* expression at all, though there is a perceptible peak during daylight hours as well. Based on transcript accumulation alone, this suggests that *FrCO*, like *AtCO*, is a promoter of flowering, and that the lack of flowering under long days does not necessarily derive from an inhibition of flowering, but instead from a lack of promotion, and possibly inhibition through other mechanisms. Interestingly, even the DN cultivar, Diamante, displayed this pattern, suggesting that the critical difference between SD and DN cultivars is not one of *CO* expression. If so, then flowering under LD conditions in Diamante must be the result of elements further down the pathway than *FrCO.* Expression of *FrCO* in 'Diamante' was slightly different than the other cultivars, with a somewhat flatter, wider peak. In light of the evidence that one of the two *FraCO* alleles in 'Strawberry Festival' is being expressed at greatly reduced levels, while 'Diamante' expresses both alleles robustly, this broad peak may be the result of two or more alleles expressed somewhat out of phase with each other, with the overlapping curves giving the impression of a wider peak.

Some caution is warranted in the interpretation of these results, as this data reflects only mRNA transcript levels. Analysis of transcript levels does not take into consideration the mechanisms that regulate post-translational regulatory mechanisms. Elegant studies by Valverde et al. (2004) demonstrate that light-quality driven alterations in CO stability and localization are critical components of CO action.

The expression pattern seen in strawberry does not appear to have been previously described, and may represent a fundamental change in the perceived function of *CO*. In Arabidopsis and rice, according to the external coincidence model, flowers are initiated or inhibited in response to whether the peak of *CO* occurs in daylight or darkness. Without knowing the dynamics of protein level, it is difficult to say for certain what the mechanism is here, but it appears that the critical factor is not one of when the peak in *CO* occurs, but how high it is. Whether this trend seen at the transcript level is continued at the protein level, where further regulation may occur (Valverde et al., 2004), remains to be seen. If it is, then some factor other than the integration of photoperiod and circadian cycle provided by *CO* is likely responsible for the perception of photoperiod in strawberry, at least with respect to flowering.

CO transcription is regulated by a number of factors, and a switch between SD and LD flowering in eudicots may be a result of a change in one of these elements, rather than a change in the functioning of CO itself. The evidence available does not seem to clearly indicate a mechanism for the reduced *FrCO* expression seen under LD, but several mechanisms that either reduce *CO* transcription or encourage degradation of the transcript have been identified. A probable blue-light photoreceptor, FKF1, appears to regulate *CO* transcript level by repressing CDF1, a repressor of *CO* transcription (Imaizumi et al., 2003). Other possibilities include the Group III *COL* proteins, as both AtCOL9 (Cheng and Wang, 2005) and AtCOL10 (Cheng and Wang, 2006) have been shown to decrease *CO* transcript level in Arabidopsis overexpressing those genes.

The new evidence also calls into question the role of phytochrome in the regulation of flowering, and the degree to which it is maintained across species. In Arabidopsis and other LD plants, far red-enriched extensions of day length at the end of the day accelerate flowering,

whereas red light extensions delay flowering (Kadman-Zahavi and Ephrat, 1974). This has been explained by Valverde et al. (2004), who found that phytochromes mediated the stability of the *CO* protein, with phyB, activated under red light, encouraging the degradation of the protein in the morning, and phyA, activated under far-red, stabilizing the protein. The phyB receptor also regulates flowering through the upregulation of PFT1, a repressor of FT (Halliday et al., 2003) as well as possibly promoting the expression of *LFY* (Blázquez and Weigel, 1999).

While this model explains the flowering responses to light quality observed in LD plants, it becomes clear that something different is happening in SD plants. Vince-Prue and Guttridge (1973) found that just the opposite occurred in strawberry, with far red extensions inhibiting flowering if applied at the beginning of the day, and red light extensions inhibiting if applied at the end of the day. Kadman-Zahavi and Ephrat (1974) grew plants under blue light supplemented with far red, and found flowering delayed compared to blue light alone or shaded conditions. While this might seem consistent if *FrCO* was a repressor of flowering, the expression data seen in our study would seem to suggest that it is not. In fact, rice, in which *CO* actually has been determined to be a repressor of flowering, does *not* display this pattern, but instead reacts in the same manner as LD plants (Kadman-Zahavi et al, 1976). Because most of this regulation is occurring at the level of the protein, it is difficult to gauge what is occurring in strawberry based solely on our transcript level data.

Kadman-Zahavi and Ephrat (1974) described two clear groups of SD plants, based on this reponse. With strawberry, they place *Chrysanthemum morifolium* Ramat. and *P. nil*, while in the group of SD plants that respond like LD plants to red and far-red light they place *Mimosa pudica* L*, Amaranthus* sp., and *Cosmos bipinnatus* Cav.. Descriptions from earlier work also suggest that *Salvia occidentalis* Sw. is also similar to strawberry (Meijer, 1959), whereas corn (*Zea mays*

L.), rice, and sorghum (*Sorghum vulgare* L.) belong to the other group (Kadman-Zahavi et al, 1976; Lane, 1962). Roses, close relatives of strawberries, have been shown to have an increase in flowering in response to red light extensions at the end of the day (Maas and Bakx, 1995, 1998), so it appears both groups may exist even within the Roisoideae. If so, this may imply a relatively simple difference in the mechanisms controling such responses.

Expression of Other COL Genes

Despite belonging to a common subgroup, *FraCOL1* and *FraCOL2* displayed rather different expression profiles. *FraCOL2* appears to be expressed in a pattern similar to *FraCO*, a pattern previously shown to be common to other *COL* group Ic genes in Arabidopsis. Microarray data by Smith et al. (2004) show that *AtCOL3* and *AtCOL4* follow similar diurnal patterns, with peaks around dawn under long days. Most other *COL* genes that have been investigated, including *AtCOL1*, *AtCOL2,* and *AtCOL9* (Ledger et al. 2001, Cheng & Wang, 2005) also show this pattern.

The variation in splicing efficiency of *FraCOL2* does not seem to follow a clear pattern, however it may serve as a means of negative regulation of its function, as the introns in *F. nubicola* FDP601, *F. vesca* FDP815, and *F. vesca* Hawaii 4 all contain multiple stop codons that would result in a truncated protein. Such a truncated protein would lack the M4 and CCT domains. Little is known about the functions of these elements. The CCT domain has been proposed to be involved in the localization of the protein; however the *co* mutants *co-5* and *co-7*, with mutations in the CCT domain, localize correctly but do not function properly (Robson et al., 2001). Given that the function of *FrCOL2* is unknown, it is difficult to speculate on the role of this truncated protein, if any, but it seems conceivable that it might compete with the functional version of the protein in its interactions with either DNA or protein.

Splice variants of the *CO* ortholog *PnCO* of Japanese morning glory were documented by Liu et al. (2001), who noted both unspliced and alternatively spliced transcripts in addition to the correctly spliced version. A majority of transcripts contained the full intron, whereas smaller numbers were properly spliced or retained a small 26 bp segment of intron sequence. The partial retention of the intron resulted in a frameshift and premature stop codon, but the presence of the entire intron caused the coding sequence to remain in the correct reading frame. Despite this, only the properly spliced transcript was able to complement Arabidopsis *co* mutation in transgenic plants. The flowering-related gene *Proliferating Inflorescence Meristem (PIM)* from pea, a homolog of the Arabidopsis meristem identity gene *AP1,* has also been shown to commonly exist as an unspliced transcript (Taylor et al., 2002).

Transcripts with unspliced introns are moderately common in plants (Ner-Gaon et al., 2004). Alexandrov et al. (2006) found that about 7% of transcripts demonstrated some sort of alternative splicing, of which 27% (Alexandrov et al., 2006) to 38% (Iida et al., 2004) contain one or more unspliced introns. Ner-Gaon et al. (2004) found that transcripts associated with physiological flux were more prone to intron retention than those involved in functions such as metabolism and housekeeping. Iida et al. (2004) found that retained introns were more common in plants that have received a recent stress, such as cold, heat, ultraviolet light, dehydration, and various hormone treatments. By producing non-functional truncated products or, alternatively, products that are degraded by nonsense-mediated decay (NMD) caused by frameshifts (Nar-Gaon et al., 2004), an extra level of regulation in genes that are critically sensitive to changes in transcript level may be provided.

Unlike *FraCO* and *FraCOL2, FraCOL1* transcript appears to remain at relatively constant levels through the course of the day. It is the only *FraCOL* gene expressed in all cultivars at the 5 pm time point under short days, the apparent low point of the *FraCO* cycle.

In contrast, the closest Arabidopsis homologs, *AtCOL3* and *AtCOL4* are both expressed in a diurnal fashion (Smith et al., 2002), although Jeong et al. (1999) did not investigate diurnal expression of the apple homologs of the *FraCOL1* gene that they characterized, nor is it clear at what point or points in the diurnal cycle tissue was collected. While both genes were expressed at the same relative level in all tissues, *MdCOL2* was consistently expressed at a higher levels than *MdCOL1*. And while most tissues showed a uniform but low level of expression, higher expression of both genes was seen in developing fruit and flower tissue, which may partly explain the great number identified in apple and peach EST libraries, because many of these were derived from fruit tissue. In strawberry, however, the majority of ESTs are from libraries derived from developing seedlings not yet producing flowers or fruit, so bias in tissue of origin cannot entirely explain the preponderance of this group among Rosaceae ESTs in GenBank.

Datta et al. (2006) oberseved decreases in the lateral branching of inflorescences in *Atcol3* mutants, and found evidence that *AtCOL3* promotes the formation of branches and inhibits the growth of the primary shoot during short days. If *FraCOL1* has a similar function in strawberry, it may be that selection for increased numbers of inflorescences or proliferation of branch crowns has selected for a mutation that results in continual expression of this gene, rather than diurnal fluctuation.

Similarly, it is also possible that the elevated expression levels for *FraCOL1* and the two *Malus* genes seen in fruit and flower tissue derives from a role within infloresence development, and that selection for increased flower number might have encouraged selection for relatively

continuous expression. It would be interesting to examine the expression of this gene in *F. daltoniana*, a strawberry species characterized by single flowers (Sargent et al. 2004b). Datta et al. (2006) demonstrated the necessity of a pair of amino-acids, VP, near the C-terminal, for binding with COP1 and conferring normal red-light phenotype. *FrCO, FrCOL1,* and *FrCOL2* all possess this motif, but it is lacking in *FrCOL3* (the C-terminus of *FrCOL4* is currently uncharacterized, although group III genes in rice and Arabidopsis lack this pair), suggesting possible roles for these first three, at least, in photomorphogenic development.

FraCOL3 was not expressed at perceptible levels in any of the RT-PCR experiments, though we were able to amplify a weak band from 'Strawberry Festival' flower cDNA. *FrvCOL3* occurs several times among *Fragaria* EST sequences, and it is worth noting that all of these are from developing seedlings, although there are only a relatively small number of ESTs available from mature plants. In *Malus* and *Prunus*, *FrCOL3*-type transcripts occur several times in mature and seedling material. Two Arabidopsis orthologs, *AtCOL6* and *AtCOL7*, coincide with QTLs for rosette leaves at flowering, days to budding, and days to flowering, suggesting possible roles in flowering, although they share this interval of the chromosome with other flowering and development related genes such as MADS-Box transcription factors and genes involved in meristem development and hormone synthesis (Bandaranayake et al., 2004).

Materials and Methods

Plant Material

Six octoploid cultivars of *F.* ×*ananassa* were used during the course of the work. These cultivars and the sources from which they were obtained are given in Table 3-5. All were obtained as rooted plants but were propagated as needed throughout the course of the experiments. Only the second group of 'Diamante' was used directly from the nursery; all others were multipled by runners in the growth chamber prior to experiments. *F. vesca* Hawaii-4 was

obtained as runner plants from the collection of Dr. Thomas Davis at the University of New Hampshire and propagated by runners in the laboratory for use in the experiments.

Plant Growth Conditions

Plants were maintained on shelves in the laboratory at room temperature (23°C), under cool white fluorescent lighting when not in use in diurnal experiments. The first diurnal experiment, utilizing 'Strawberry Festival' and 'Diamante', was conducted in the growth chamber in enclosed compartments, under either 8 h or 16 h photoperiods under cool white fluorescent lighting. Each treatment consisted of three 25 cm pots per genotype, containing four mature plants in ProMix BX soilless medium, and the pots randomized within the compartment. Temperature was maintained at approximately 23°C.

The second diurnal experiment was conducted in growth chambers, with six plants in each treatment of the octoploid cultivars Earliglow and Diamante, and two each of Camarosa. Ten plants of *F. vesca* Hawaii-4 were also grown in each chamber. Plants were grown in 10-cm square pots in ProMix BX soilless medium and watered and fertilized as needed. Once again, 8 h and 16 h photoperiods were used, with a photon flux of approximately 300 $\mu mol \bullet s^{-1} \bullet m^{-2}$ at the level of the soil surface. Photon flux was found to be fairly uniform through the chamber. Three flats containing two of each *F.* x *ananassa* cultivar were arranged in the center of the chamber, and the arrangements of the pots within each flat randomized. *F. vesca* plants were placed together in a fourth flat. Temperature was set at 18°C day / 16°C night. Plants in both experiments were watered and fertilized as needed. Total number of runners and inflorescences were counted on each plant at 4, 6, and 8 weeks from the beginning of the light treatments, except for the *F. vesca*, for which data was collected only at 6 and 8 weeks.

Cloning of *FraCO*

A partial EST sequence (CO380854) resembling *CO* was identified among sequences derived from the octoploid 'Queen Elisa'. A single primer based on this sequence, FaCOtop (5'-TGGATGTTGGAGTTGTACCAG-3') was used along with the M13 reverse primer to amplify a product from a mixed tissue 'Strawberry Festival' cDNA library by PCR. The product generated was cloned and sequenced, and a second primer, FaCO-R (5'-CGGCATTGTTCCTTCATACTAA-3') was developed. FaCOtop and FaCO-R were used to amplify a 344 bp product for use as a probe, using Touchdown PCR as described in Sargent et al. (2004). This probe was hybridized against approximately 20,000 colonies of a *F. x ananassa* 'Strawberry Festival' flower tissue library in *E. coli* /Gateway vector.

Identification of COL Genes in Genebank

One *Fragaria COL* gene, *FraCOL1*, had previously been identified in the description of an earlier EST library (Folta et al., 2005). All others were identified by TBLAST-N searches (Altschul et al., 1997) of *Fragaria* ESTs in GeneBank, using each Arabidopsis *COL* gene as the query, as well as searches using only *AtCO* CCT domain and B-Box region. Identified *Fragaria* sequences were also searched against *Malus*, *Prunus*, and *Rosa* ESTs to identify other Rosaceae homologs.

Gene Structure Characterization

Primers flanking the full coding region for each of the *FraCOL* genes were designed, as well as a primer set (RN) enclosing a roughly 400 bp segment of the coding region believed to include the putative intron site (Table 3-2). This segment of each gene was amplified from genomic sequence using Touchdown PCR (as Sargent et al. 2004, except extension time was increased to 1 min) from 'Strawberry Festival', FDP601, and FDP815, and from 'Strawberry Festival' flower cDNA. The presence of intron sequence was verified by comparing the fragment

size generated from genomic sequence to that generated from cDNA and the size predicted from the EST or ESTs that it was identified from. Combinations of the flanking and RN primers were used to confirm the absence of other introns.

Southern Blot Analysis

Total DNA was extracted from of *F.* ×*ananassa* 'Strawberry Festival' and *F. vesca* Hawaii-4 using a modified cold CTAB method (Tombolato et al., in preparation). Ten µg of each were digested with four restriction enzymes: *Eco*RV, *Hind*III, *Bam*HI, and *Eco*RI. Digested samples were separated by size using gel electrophoresis on 0.8% agarose gels, blotted onto nylon membranes, and UV cross-linked. A *FraCO* probe was developed using the RN-*FrCO*-F and *FrCO*-full-R (Table 3-2) with 'Strawberry Festival' genomic DNA in a touchdown PCR reaction as described above. The probe was labeled by random priming as per manufacturer's instructions, hybridized overnight at 60°C, and given three washes of 20 minutes each with with a wash buffer comprised of 1x SSC and 0.1% SDS.

Genetic Linkage Mapping COL genes in *Fragaria*

FrCO was mapped in a diploid reference population developed by Sargent et al. (2004, 2006). This is a F2 population from the cross of *F. vesca* FDP815 x *F. nubicola* FDP601, and consists of 94 individuals. The RN-FrCO primer set (Table 3-2) was used to amplify a product by PCR from each individuals DNA (30 cycles, 52°C annealing, 1 min extension). Ten µl of each PCR product was digested in a reaction with 0.3 µl of *Hind*III enzyme, 1.3 µl 10x bovine serum albumin, 1.3 µl 10x Promega Buffer B, and 0.3 µl dH_2O. Digested product was separated by gel electrophoresis on 1% agarose gels, stained with ethidium bromide, and photographed under UV light. FDP601 alleles displayed three bands when digested, whereas FDP815 displayed only two. Size differences allowed the trait to be scored as a co-dominant marker.

Attempts to map the *FrCOL2* locus were performed as above, with the following exceptions: the RN-FrCOL2 primer pair (Table 3-2) was used, extension time was set at 45 s per cycle, and PCR products were digested with *Afl*III enzyme. When this showed an unusual segregation pattern, the process was repeated with the *Eco*RV enzyme. In both cases, the FDP815 allele was not cut by the enzyme, whereas the FDP601 allele was. However, due to incomplete digestion, it was scored as a dominant marker.

Marker data was entered into the JoinMap 3.0 software program and mapped relative to marker data provided by Dan Sargent, consisting of most of the markers appearing in Sargent et al., 2004 and Sargent et al., 2006.

Extraction of Nucleic Acids

Except as noted above for use with the Southern blot, DNA was extracted using the Qiagen DNEasy Plant DNA extraction kit, according to the manufacturer's instructions. Some genomic DNA was provided by others, namely that of *F. vesca* Hawaii-4, *F. iinumae* FRA377, *F. bucharica* FRA520, and *F. mandshurica* FME, from Dr. Thomas Davis at the University of New Hampshire, and *F. vesca* FDP815 and *F. nubicola* FDP601 and that of the F2 mapping population from these parents from Dr. Daniel Sargent of East Malling Research.

RNA was extracted using a modification of the pine cone method of Chang et al. (1993) as described in Folta et al. (2005). Briefly, 1 g of tissue was frozen with liquid nitrogen and ground with a mortar and pestle, then incubated at 65°C for 10 min in a CTAB-based extraction buffer (2% CTAB, 2% polyvinylpyrrolidone, 100 mM Tris-HCl (pH 8.0), 25 mM EDTA, 2.0 M NaCl, 0.5 g/ml spermidine, and 2.0% beta-mercaptoethanol). Samples were then allowed to cool to room temperature and equal volumes of chloroform:octanol were added and the samples homogenized using a Polytron T10-35 tissue homogenizer at 90% of full speed. The organic and aqueous phases were separated via centrifugation at 8,000 x g. The supernatant was then

removed, mixed with equal volumes chloroform:octanol, and then separated via centrifuge again. LiCl was added to the resulting supernatant to a concentration of 2.5 M and allowed to precipitate overnight at 4°C, after which it was centrifuged again at 10,000 x g. The pellet was resuspended in 500 µl SSTE (1 M NaCl, 0.5% SDS, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA) and again purified with chloroform:octanol, then precipitated with two volumes of 100% ethanol. The pellet was then washed with 76% ethanol, 0.3M sodium acetate, dried using a SpeedVac, and resuspended in 50µl of 10 mM Tris-HCL (ph 8.0), 2.5 mM EDTA. Samples were quantified by spectrophotometry.

Reverse Transcription and RT- PCR

For the first photoperiod experiment 1 µg of RNA from each condition was reverse transcribed using AMV reverse transcriptase (Promega Inc., Madison, WI) as per the manufacturer's protocol. The resulting cDNA was diluted 1:10 with TE buffer and used as template for RT-PCR reactions.

Initial experiments were conducted with the 'Camarosa' SD cDNA set to determine the likely linear range for each pair of primers. For RN-FrCO, RN-Ubiq, and RN-Actin primers sets, reactions were conducted at 24, 28, 32, and 36 cycles, and the highest number of cycles which retained the relative band intensities seen at lower numbers of cycles was selected. RT-PCR reactions used the RN primer sets described in Table 3-2 using standard 3 step PCR (52°C annealing temperature, 30 seconds extension, for 32 cycles for RN-FrCO and RN-Actin, 28 cycles for RN-Ubiq), separated by electrophoresis on 1% agarose gel, then stained with ethidium bromide and photographed under UV. Template amounts were adjusted using ubiquitin and actin RN primer sets (Table 3-2) to assure similar amounts of template DNA between cultivars and treatments and the PCR repeated and gels photographed (Figure 3-13). Allele-specific RT-PCR trials used the same PCR conditions, but 33 cycles was used because slightly less product was

anticipated than for with the RN-FrCO primer pair. A second round was conducted at 40 cycles, with the idea of reach saturation and testing the whether any *FrCO-C9* product was present in 'Strawberry Festival'.

The second experiment used a similar procedure, but reverse transcription was done using ImProm II reverse transcriptase (Promega Inc., Madison, WI) as per manufacturer's instructions. The resulting 20 µl reaction was not diluted but used directly as template in a PCR reaction identical to above, except that 35 cycles was used for *FrCOL2*. Template volume used in each reaction was adjusted using ubiquitin and actin RN primers to equalize template amounts. The products were again separated by electrophoresis (on a 1.5% agarose gel), stained and photographed.

Table 3-1. Comparison of amino acid identity (%) of the predicted protein encoded by FraCO to those of Group Ia CONSTANS-LIKE genes in apple (*Malus domestica*), castor bean (*Ricinus communis*), Cottonwood (*Populus deltoides*), thale cress (*Arabidopsis thaliana*) rape (*Brassica napus*), Japanese morning glory (*Pharbitis nil*), rice (*Oryza sativa*), wheat (*Triticum aestivum*), and perennial ryegrass (*Lolium perenne*).

Gene	Acc. No.	B-Box 1	B-Box 2	M1	M2	M3	M4	CCT	Overall
MdCO	[z]	95	97	92	52	80	100	97	75
RcCO	[y]	83	92	92	47	70	100	97	71
PdCO1	AY515150	86	100	92	68	90	80	93	69
AtCO	NM121589	76	82	60	63	60	60	88	54
PnCO	AF300700	74	82	78	47	55	80	97	56
BnCOa1	AY280868	74	74	45	57	70	30	88	50
OsHd1	AB041840	76	80	42	58	50	60	88	50
TaHd1-1	AB094490	67	75	33	22	50	50	88	43
LpCO	AY553297	67	51	58	47	50	50	84	41

[z] *Malus ×domestica CO* gene assembled from ESTs EB130204, EB141442, and DR996733

[y] *Ricinus communis CO* gene assembled from ESTs EG683156 and EG686997

Table 3-2. RT-PCR primers for strawberry CONSTANS-like genes and controls, sequence source, T_M, and approximate size in cDNA (as calculated from sequence).

Gene	Primers	Sequence	TM (°C)	EST	bp
CONSTANS-like Genes					
FrCO	RN-FrCO-F	ctgaatcctgtgaagaacagc	53.6	—[z]	388
	RN-FrCO-R	tggatgttggagttgtaccag	54.4		
FrCO-35	RN-FrCO-35-F	catctgatcagaaccagttca	52.1	—[z]	265
FrCO-C9	RN-FrCO-C9-F	catctgatcagaacctcg	55.3	—[z]	256
FrCOL1	RN-FrCOL1-F	ctgaggccgaggctgcttcg	63.2	CX661699	403
	RN-FrCOL1-R	ctgtaccttaacaccctggc	55.3	DY674862	
FrCOL-7C	RN-FraCOL7C-F	gatggcaacatgctgacggac	59.0	CO817106	117
FrCOL2	RN-FrCOL2-F	tgatgccctcgacatgaagc	57.6	DY669887	411
	RN-FrCOL2-R	cccggtccactccggtcagc	65.1	DY671835	(531)[y]
FrCOL3	RN-FrCOL3-F	ttccaacgctgtttccaacg	56.3	DY667001	418
	RN-FrCOL3-R	ctcagcctccatgagcaccgc	62.9	DY673134	
Control Genes					
FrActin1	RN-Actin-F	tggctgtgcacgatgattgc	58.8	DV439971	430
	RN-Actin-R	taacttcccaccagatatcc	50.9		
FrUbiq1	RN-Ubiq-F	aaccaaccgtccaacaatcccaac	60.1	CX661133	414
	RN-Ubiq-R	accggatcagcagaggttgatctt	60.0		

[z] Sequence obtained in the course of this study

[y] Approximate size in transcripts containing unspliced intron

[x] 5' portion of transcript sequenced from library using universal primers

Table 3-3. GenBank accession numbers for *Fragaria* COL genes and other *Rosaceae* orthologs.

***Fragaria COL* gene**	**Arabidopsis orthologs**	Malus *sp.*	Prunus persica	Prunus dulcis	Prunus armeniaca	***Rosa* sp.**
FrCO DY675636 DY672035 DY669597 CO380854	AtCO	DR996733 CV627730 CO754283 EB132548 EB130204 DR996467 EB141396 EB148115 EB141172 EB129058 CV998001 CV883514 EB141442 CV657475 EB141442 CV657475	DY637206 BU046876 BU046875			
FrCOL1 CX661699 CX661092 CX661512 CX662230 DY674862 CO817106	AtCOL3/4	AF052584 (MdCOL1), AF052585 (MdCOL2), and 66 ESTs	DY644780 DY643071 DY641101 DY635684 DY635102 DN555580 DN555698 BU042239 (PrpCO)		CV049551 CB821542	EC589436
FrCOL2 DY669887 DY671835 DY674801	AtCOL5	EB157350 and 20 other ESTs	BU041471 DY644780			BI977339 BI977724
FrCOL3 DY667001 DY673134	AtCOL6/16		DN554754 DN553950	BQ641156		
FrCOL4 DY674124	AtCOL9/10	EB144446 EB144014 CN581286	BU044949 DW351298			BQ103878

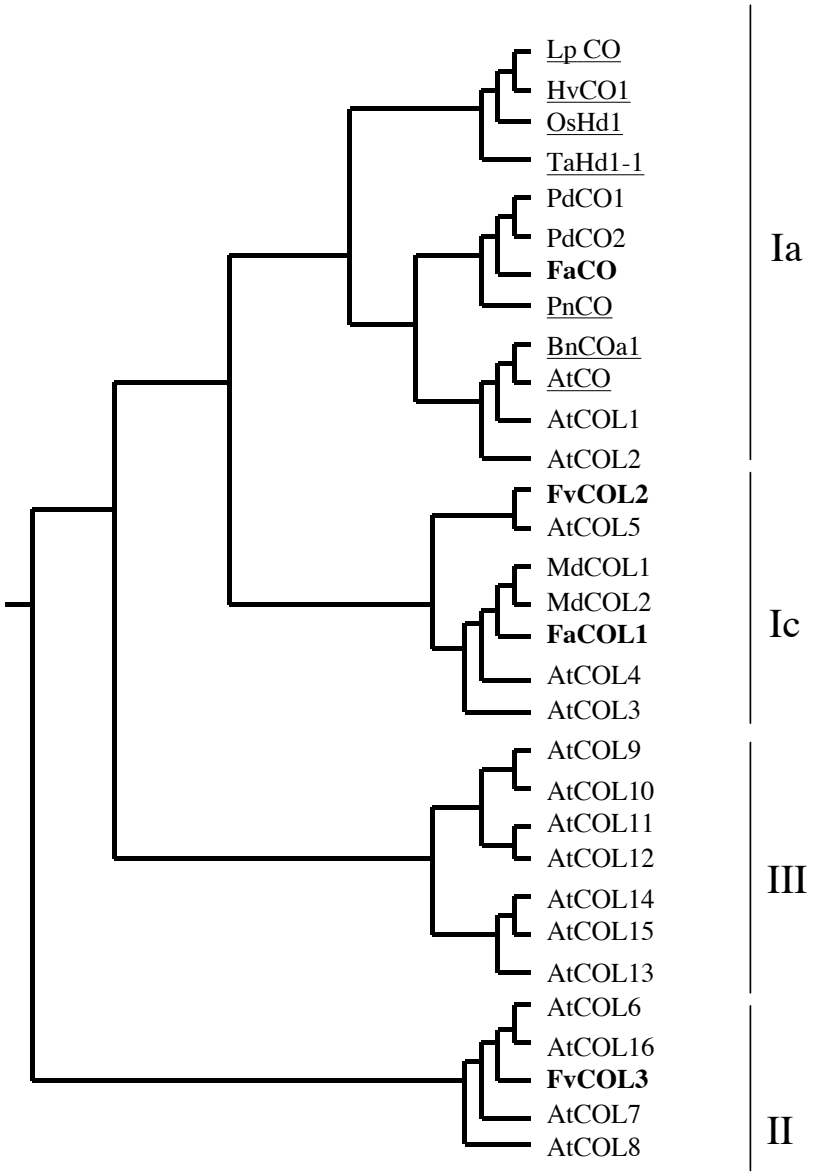

Figure 3-1. Cladogram of CO-like genes, including all Arabidopsis and full-length Rosaceae COL genes as well as all those from other species Group Ia genes demonstrated to functionally complement Arabidopsis *co* mutants (underlined). NJ tree was constructed based on the full-length predicted amino acid sequence of each gene. Because the full sequence was not available, *FvCOL4* is not included in the analysis, but appears most similar to *AtCOL9* and *AtCOL10.* Strawberry genes are shown in bold. Groups follow the criteria of Griffiths et al., 2003.

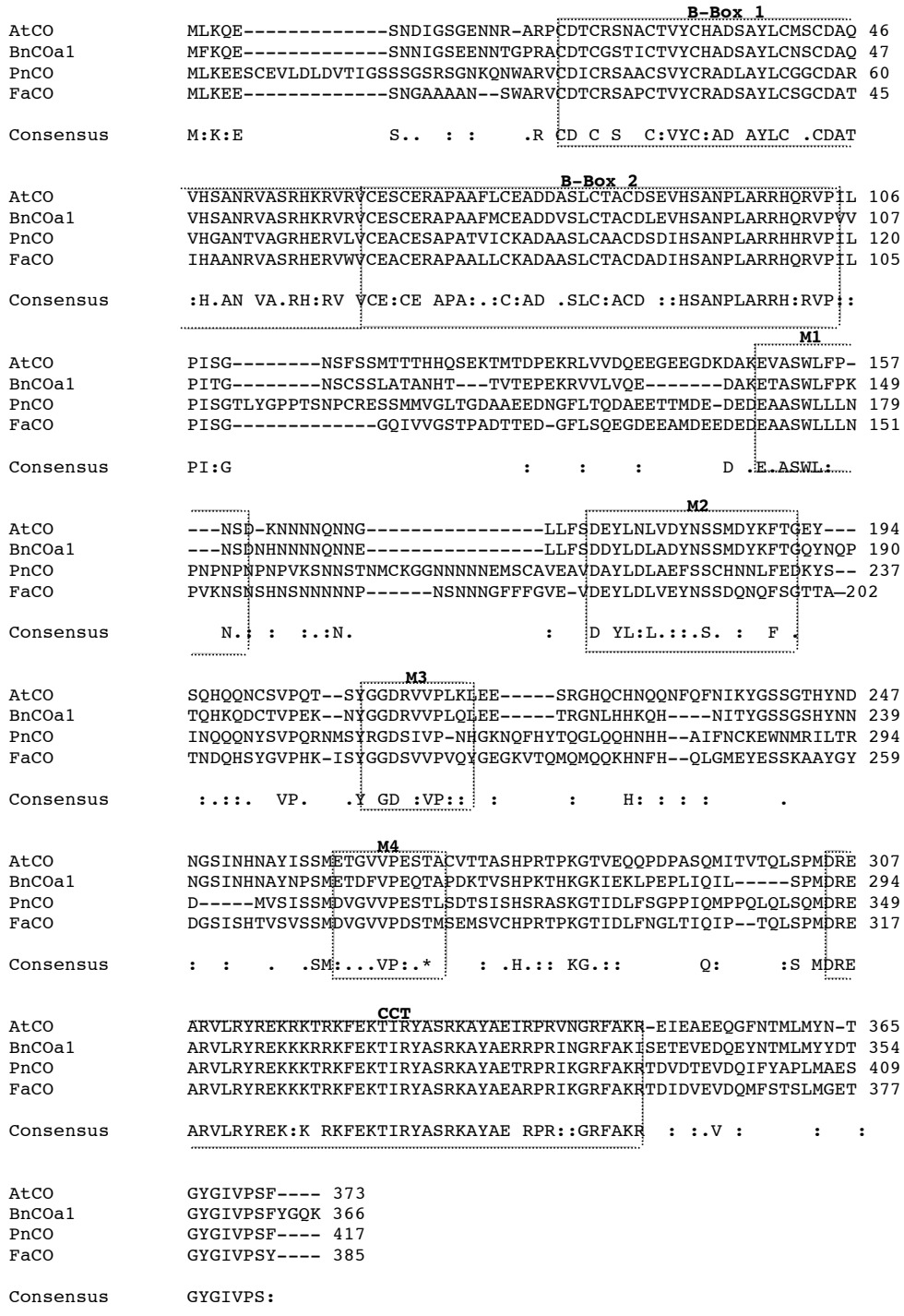

Figure 3-2. Alignment of the predicted amino acid sequence for FraCO with those of AtCO and functionally confirmed dicot homologs, with major conserved domains noted.

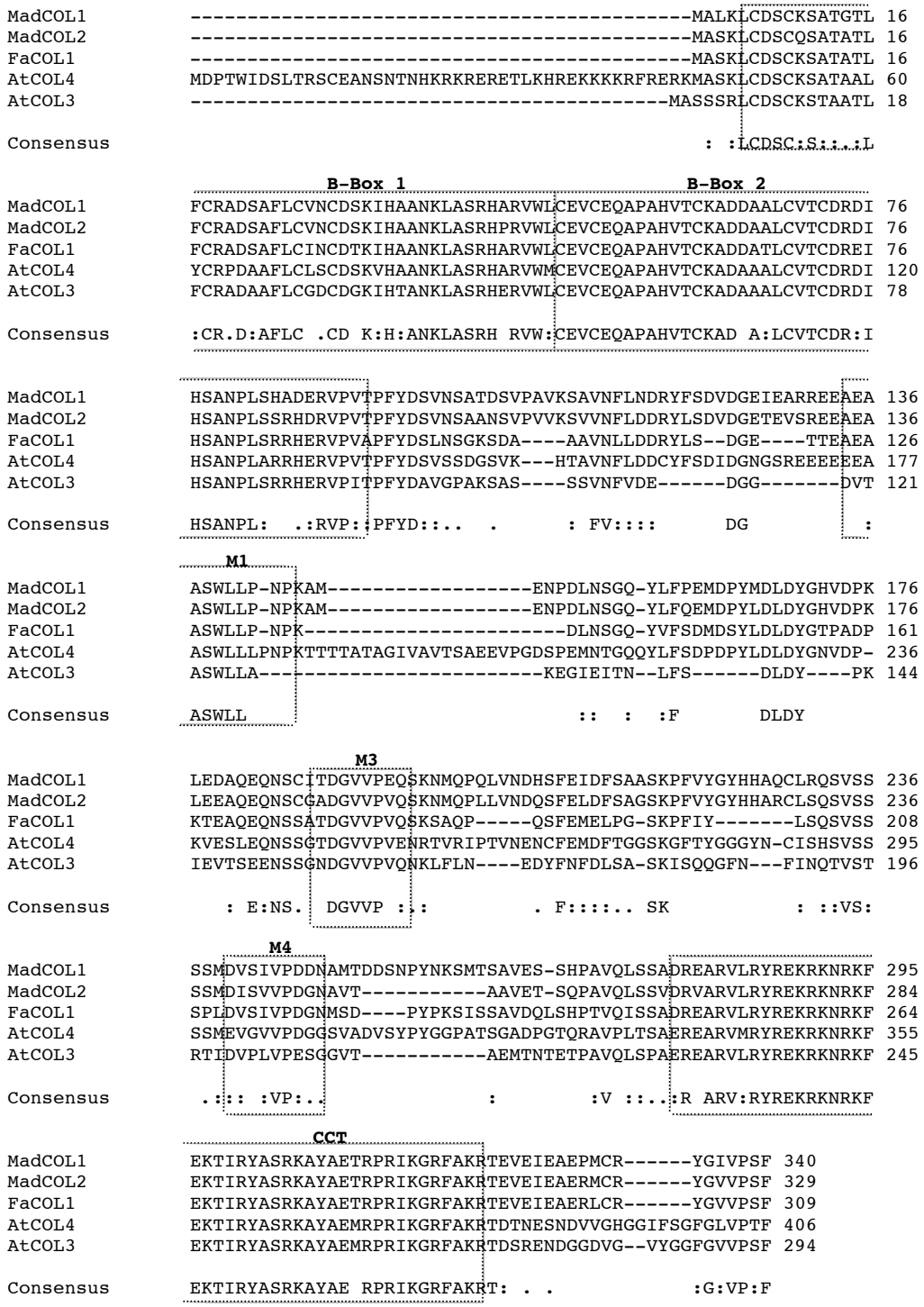

Figure 3-3. Alignment of the predicted amino acid sequence for FrvCOL1 with possible apple and Arabidopsis homologs, with major conserved domains noted.

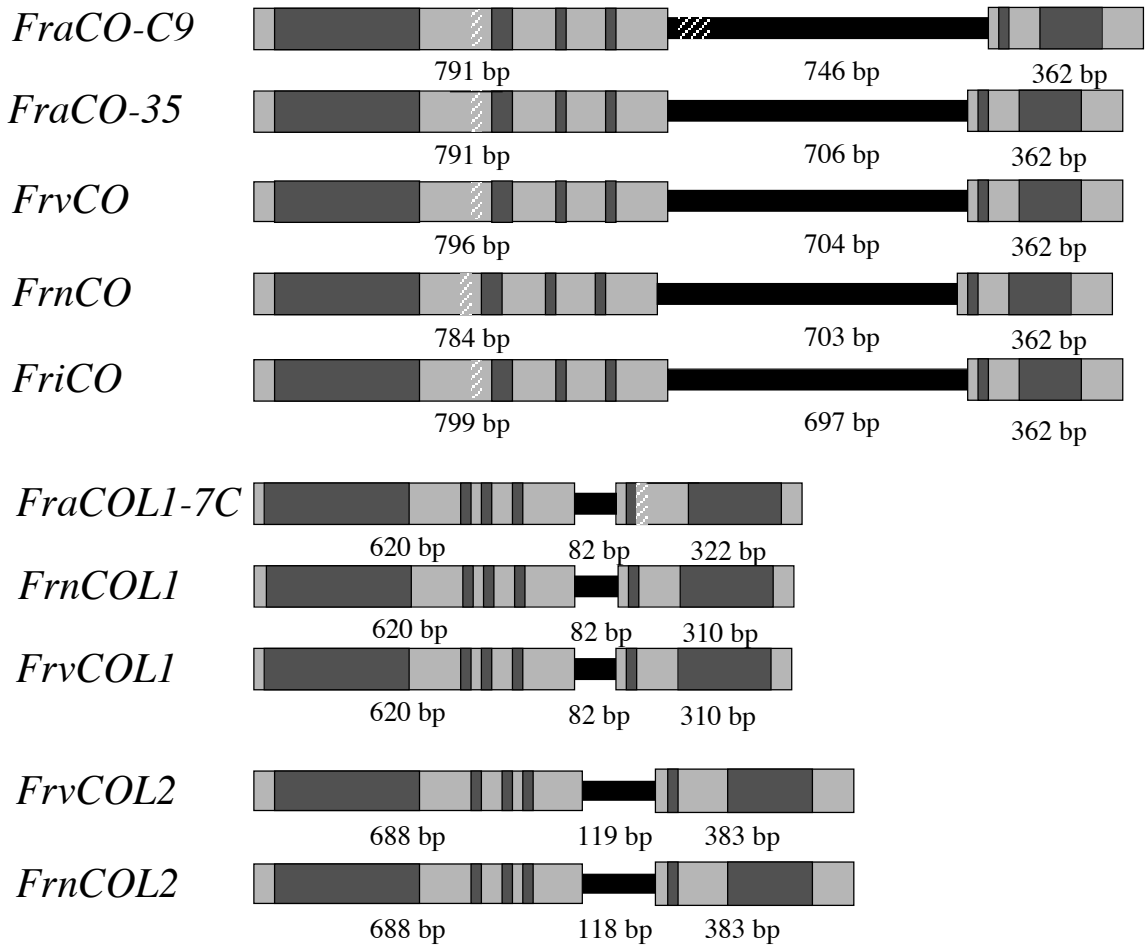

Figure 3-4. Structures of *COL* alleles from *Fragaria* species: *F.* ×*ananassa* 'Strawberry Festival' (*Fra*), *F. vesca* FDP815 (*Frv*), *F. nubicola* FDP 601 (*Frn*), and *F. iinumae* FRA377 (*Fri*). Thinner black lines indicate introns, dark grey indicates conserved domains, and crosshatching indicates major indel polymorphism sites.

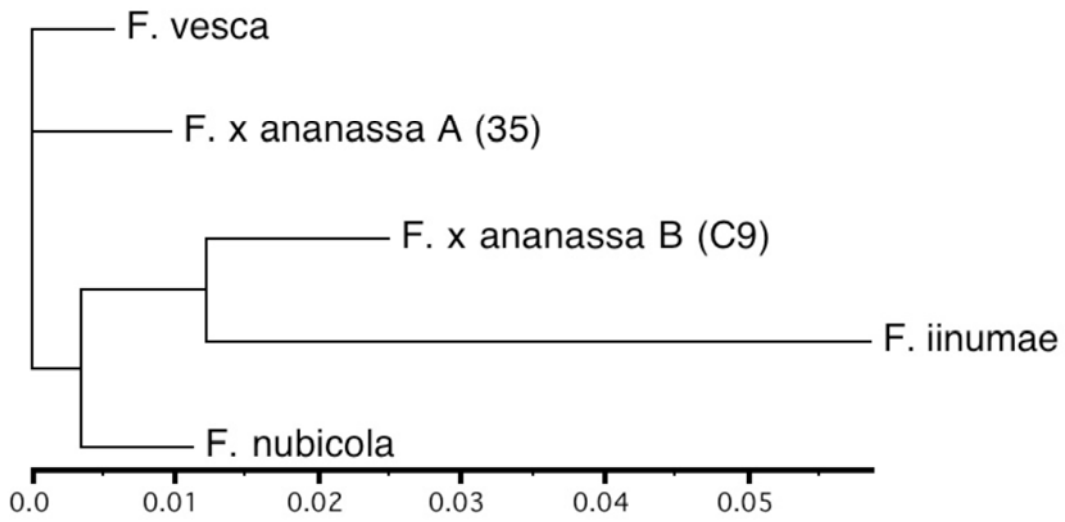

Figure 3-5. NJ phylogram tree of intron sequence divergence among *FrCO* alleles.

(A)

```
F. vesca Hawaii-4            CTGTGAA--ACAGC-AATAGCCACAACAGTAACA-ACAACAACAATCCGAACAGT-AAC
F. vesca FDP815              CTGTGAAGAACAGC-AATAGCCACAACAGTAACA-ACAACAACAATCCGAACAGT-AAC
F. nubicola FDP601           CTGTGAAGAACAGC-AATA----------------ACCACAACAATCCGAACAAT-AAC
F. x ananassa 'Festival' C9  CTGTGAAGAACAGC-AATA----------------ACCACAACAATCCGAACAAT-AAC
F. x ananassa 'Festival' 35  ATCTGAATCCTGTGAGACAGCAATAACT-------ACAACAACAATCCGAACAGT-AAC
F. bucharica FDP520          ATCTGAATCCTGTCAGGACAGCACCACGCACCAG-------TAACACACAACAATCGAC
F. iinumae FRA377            ATGTGAACGGATAACAATTTTCACACAGGAAACAGCTATTG-----ACCCATGAT-TAC
                              ******                                        *   *  **

F. vesca Hawaii-4            AACAACGGATTCTTCTTCGGAGTGGAGGTTGATGAGTACTTGGACCTTGTGGAGTACAAC
F. vesca FDP815              AACAACGGATTCTTCTTCGGAGTGGAGGTTGATGAGTACTTGGACCTTGTGGAGTACAAC
F. nubicola FDP601            AACAACGGATTCTTCTTTGGAGTGGAGGTTGATGAGTACTTGGACTTTGTGGAGTACAAC
F. x ananassa 'Festival' C9  AACAACGGATTCTTCTTTGGAGTGGAGGTTGATGAGTACTTGGACTTTGTGGAGTACAAC
F. x ananassa 'Festival' 35  AACAACGGATTCTTCTTCGGAGTGGAGGTTGATGAGTACTTGGACCTTGTGGAGTACAAC
F. bucharica FDP520          ATCCACCAACGGATCTCTCGGAGTGAGGTTGATGAGTACTTGGACCT-GTGGAGTACAAC
F. iinumae FRA377            GGCCAAGCTTGGTACCGAGGCTCCGGATTCCACTAGTAACGGCCGCCAGTGTGCTGGAAT
                               * *          *   *    *   *  *   ***   *       **   *  **
```

(B)

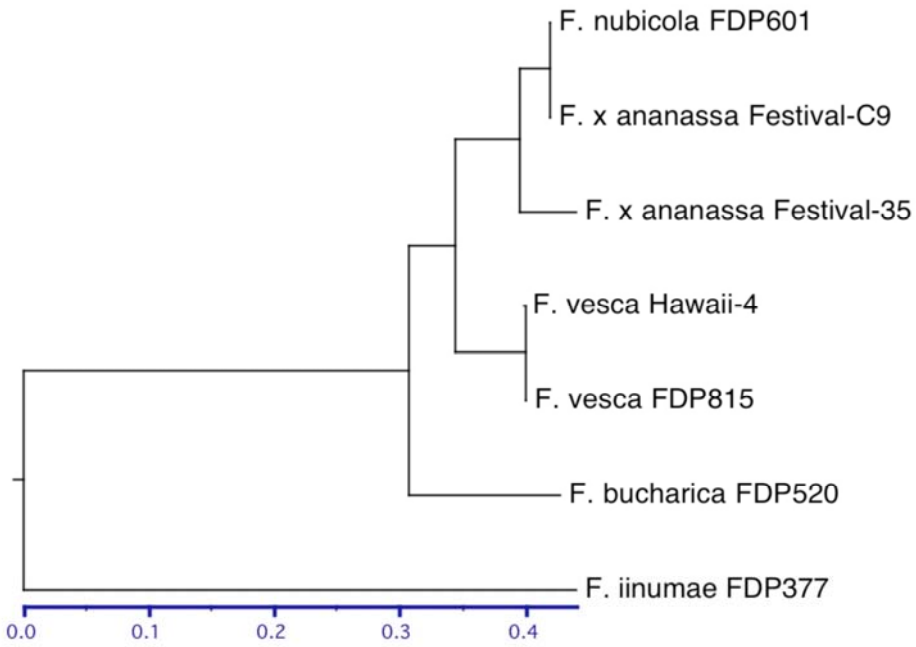

Figure 3-6. Comparisons of hypervariable section of coding region in *FrCO*. (A) Alignment of variable region of seven *FrCO* alleles, (B) NJ phylogram comparing nucleotide sequence within the variable region.

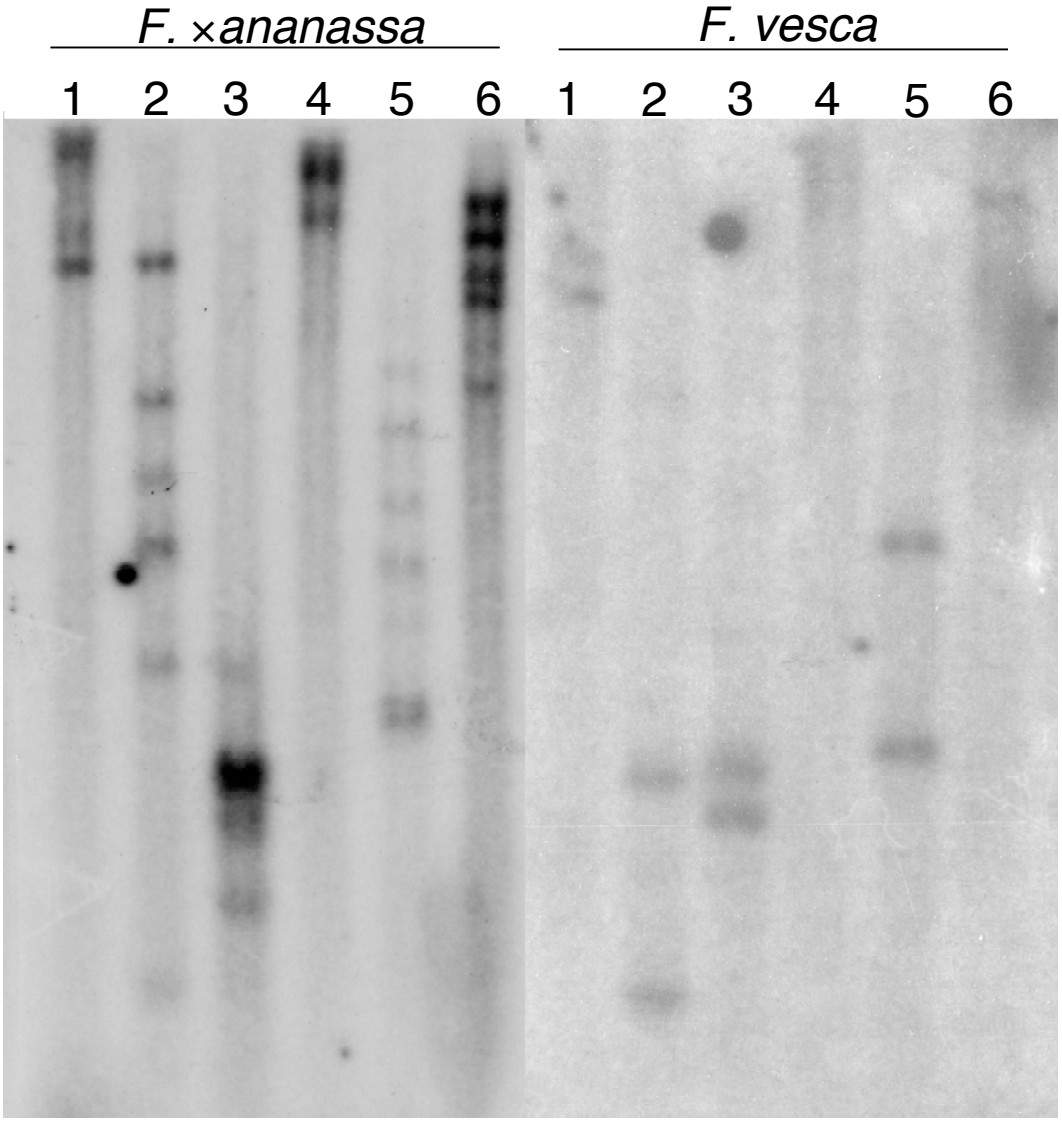

Figure 3-7. Southern blot of genomic DNA from *F.* ×*ananassa* 'Strawberry Festival' and *F. vesca* Hawaii 4, cut with six different enzymes and probed with a portion of *FrvCO*. Lane 1=*Xho I,* 2=*Xba I*, 3=*Hind III*, 4=*EcoR V*, 5=*EcoR I,* 6=*BamH I.*

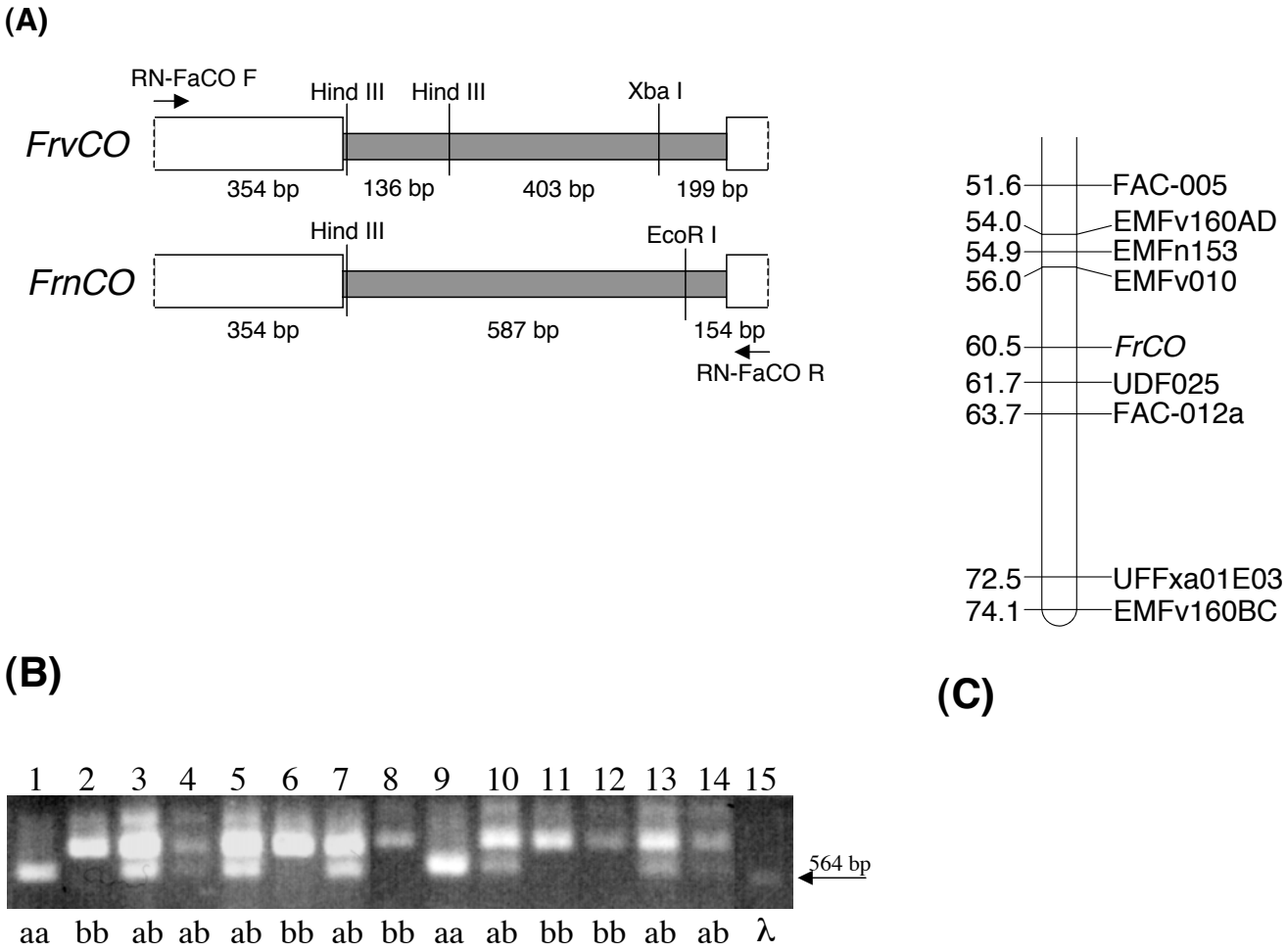

Figure 3-8. (A) Map of the portion of the *FrCO* product amplified for use in mapping, showing the locations of distinguishing restriction sites and lengths of the resulting fragments. (B) Gel showing characteristic band patterns after digestion with Hind III for the *F. vesca* parent (lane 1, genotype aa), the *F. nubicola* parent (lane 2, genotype bb), twelve members of the F_2 (lanes 3-14), and the 564 bp band of the λHind III size standard (lane 15). (C) Partial map of linkage group VI (after Sargent et al. 2005), showing the predicted location of *FrCO*.

Table 3-4. Mean inflorescence and runner production of *F.* ×*ananassa* and *F. vesca* genotypes under LD (16 h) and SD (8 h) photoperiods, at 18/16°C day/night temperature.

	4 weeks		6 weeks		8 weeks	
Genotype	**Inflorescences**	**Runners**	**Inflorescences**	**Runners**	**Inflorescences**	**Runners**
			Long Day			
Diamante	1.2	0.0	3.2	0.0	3.8	0.2
Camarosa	0.0	0.0	0.0	0.0	0.0	0.0
Earliglow	0.0	2.2	0.0	3.0	0.0	3.8
Hawaii 4	—	—	0.1	2.8	0.2	3.8
			Short Day			
Diamante	0.4	0.0	0.6	0.0	1.0	0.0
Camarosa	0.0	0.0	0.3	0.0	1.0	0.0
Earliglow	0.0	2.4	0.0	2.8	0.0	3.0
Hawaii 4	—	—	3.0	1.8	3.3	1.8

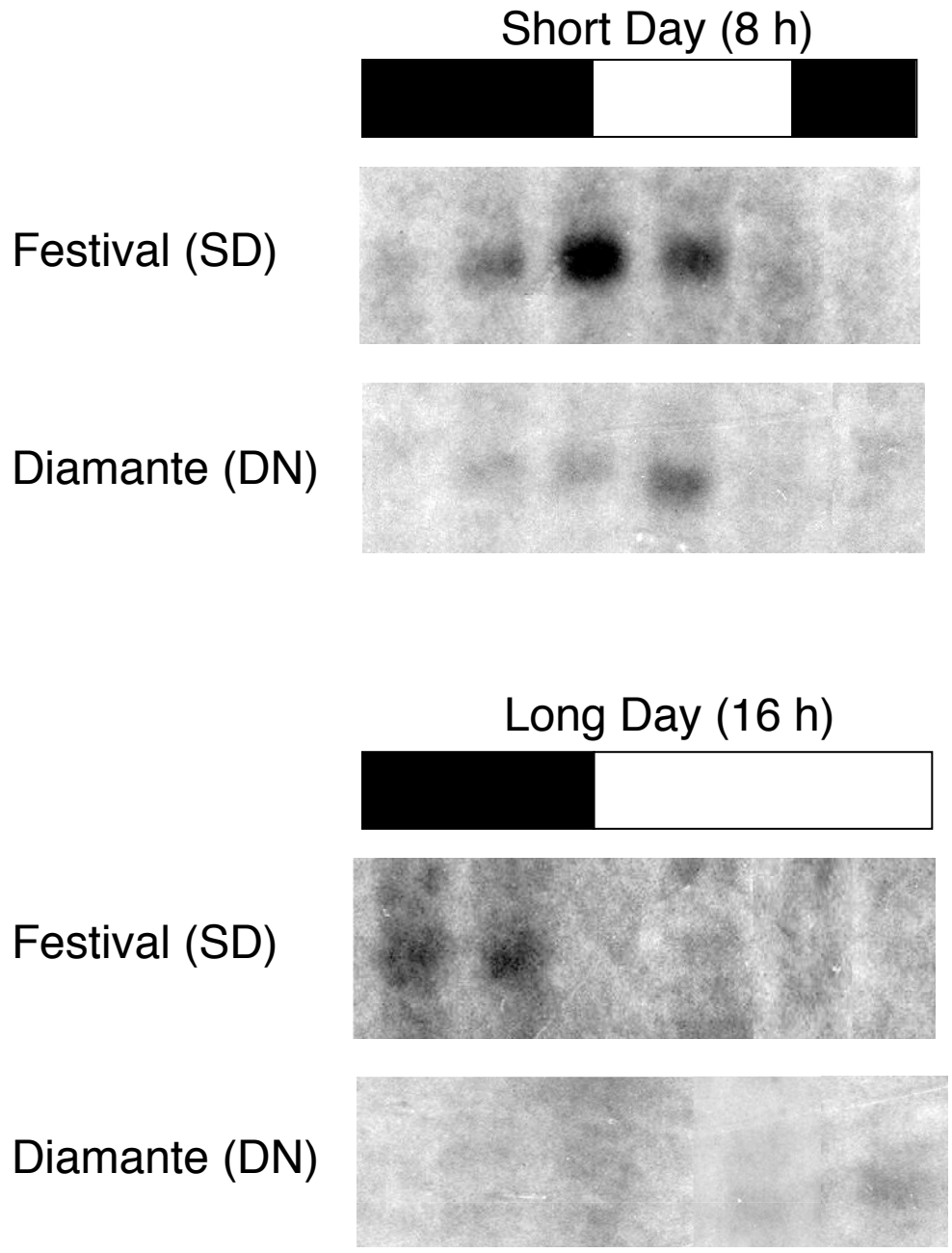

Figure 3-9. Northern blot of RNA from 'Strawberry Festival', a SD genotype, and 'Diamante', a DN genotype, collected every 4 h, showing expression of *FraCO* under short and long day conditions.

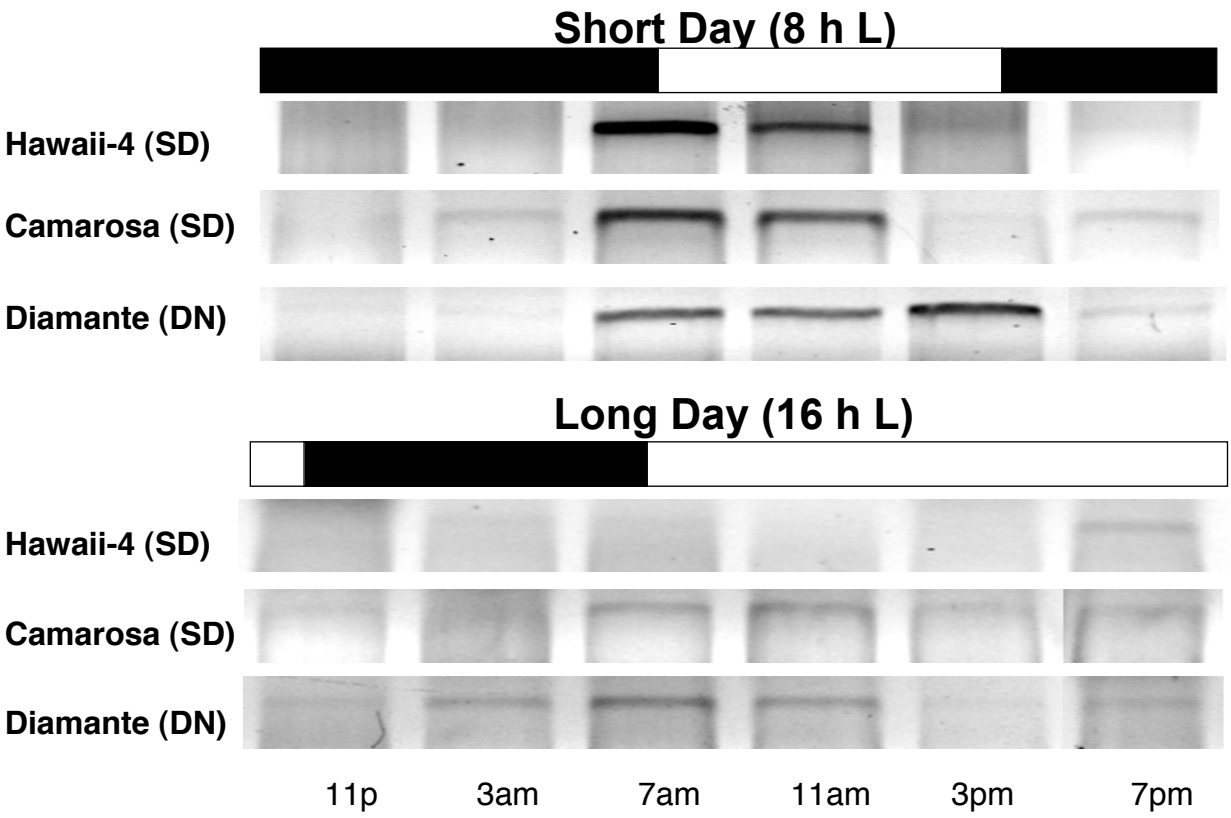

Figure 3-10. RT-PCR assay of expression of *FrCO* transcript under short (8 h) and long (16 h) days in three genotypes: *F. vesca* Hawaii-4 (SD, diploid), *F.* ×*ananassa* 'Camarosa' (SD, octoploid), and *F.* ×*ananassa* 'Diamante' (DN, octoploid). Dawn occurred at 8 a.m. in both chambers, and dusk was at 4 p.m. in the short day chamber and at midnight in the long day chamber.

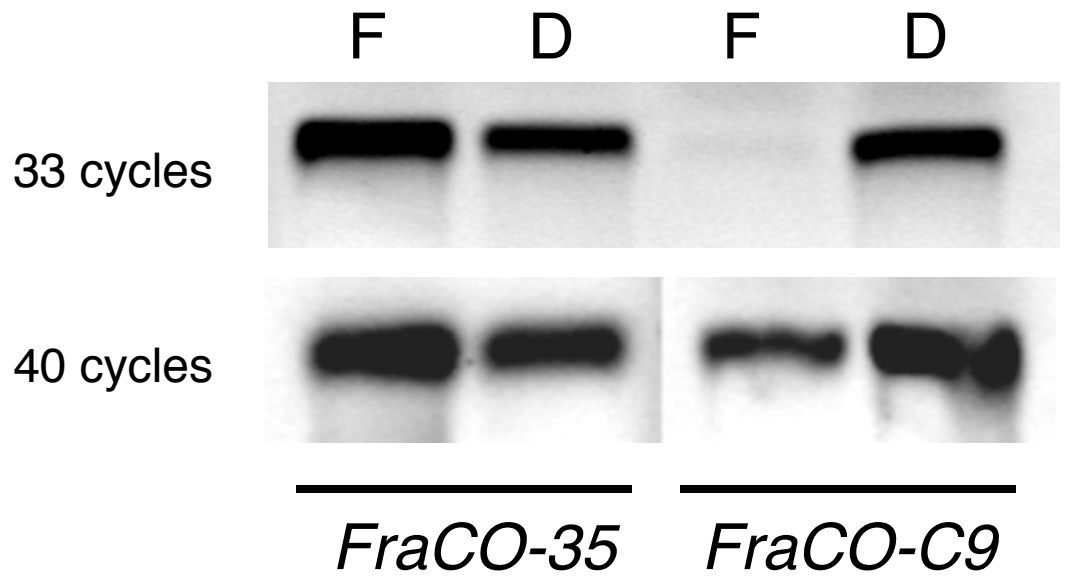

Figure 3-11. Expression of *35* and *C9* alleles of *FraCO* at dawn under SD, using RT-PCR at 33 and 40 cycles, in 'Strawberry Festival' (F) and 'Diamante' (D).

Table 3-5. Sources of plants used in this chapter.

Genotype	Species	Source	Location
Camarosa	*F.* ×*ananassa*	Garden Gate Nursery	Gainesville, FL
Earliglow	*F.* ×*ananassa*	Edible Landscaping	Afton, VA
Diamante	*F.* ×*ananassa*	Edible Landscaping	Afton, VA
Strawberry Festival	*F.* ×*ananassa*	GCREC	Wimauma, FL
Hawaii-4	*F. vesca*	University of N.H.	Durham, NH

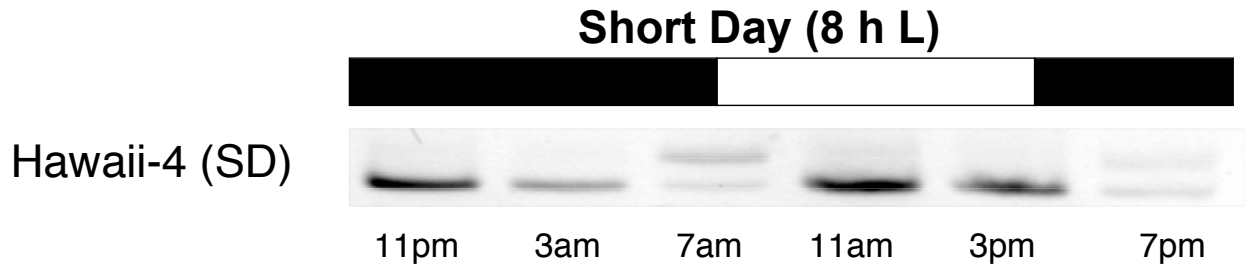

Figure 3-12. RT-PCR assay of *FrCOL2* expression in *F. vesca* Hawaii-4, showing variation in splicing efficiency at several points under short days.

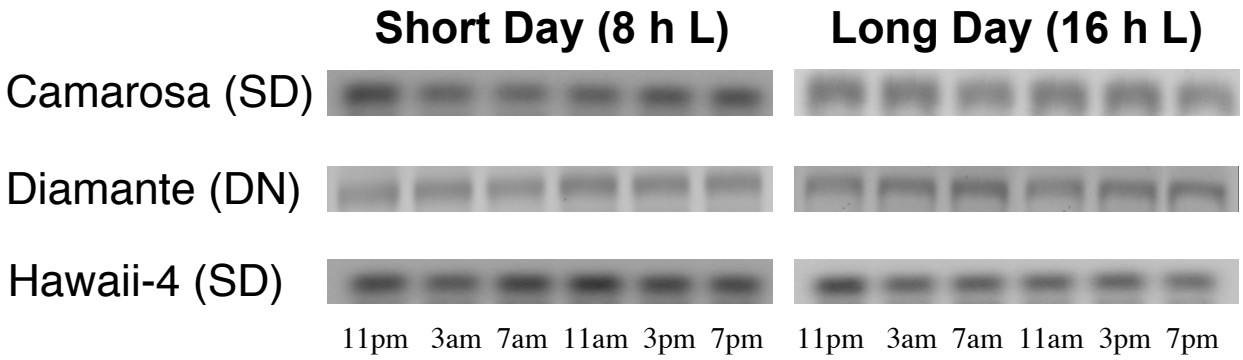

Figure 3-13. Ubiquitin controls after equalization of RT-PCR samples using RN-Ubiq1 primer set to standardize template amounts.

CHAPTER 4
IDENTIFICATION AND CHARACTERIZATION OF FLOWERING-RELATED GENES IN *Fragaria* AND OTHER ROSACEAE

Introduction

Although *CONSTANS* plays a central role in the integration of photoperiod and the circadian clock into the regulation of flowering, *CO* itself does not directly control the reproductive transition. Instead, *CO*, along with other inputs, regulates a network of proteins which through a cascade of interactions eventually trigger the activation or repression of a suite of meristem identity genes that ultimately govern the developmental fate of the meristem.

In Arabidopsis, the two most important regulators of flowering directly downstream from *CO* are *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) (Samach, 2000). These two proteins act in parallel to promote flowering, and are affected by different environtmental inputs. A third protein, *TERMINAL FLOWER 1* (*TFL1)* is closely related to *FT*, but has an opposing role. Rather than promoting a transition to flowering in the meristem, *TFL1* delays it, causing growth to remain vegetative (Kobayashi et al., 1999). *FT* and *TFL* seem to have fairly direct impacts on the meristem, but *SOC1* acts primarily through the activation of protein called *LEAFY* (*LFY*) to promote flowering. *FT* appears to be under greater photoperiodic control than *SOC1* (Moon et al., 2005). Although promoted by *CO*, *SOC1* is also under regulation by the other pathways regulating flowering: vernalization, autonomous, and gibberellin, and serves as the point at which these regulatory networks integrate into the general flowering pathway (Izawa et al., 2003).

In addition to these components, a number of other genes affect flowering as well. These include components of the circadian clock (Turner et al., 2005) and photoreceptors (Izawa et al.,

2003), which affect *CO* transcript and protein levels, as well as components of the other floral pathways.

Mutations in these genes cause significant changes in flowering time and sensitivity to environmental inputs in Arabidopsis, as well as in crop plants such as rice (Izawa et al., 2003). Although the genetic basis for variation in flowering habit in strawberry is currently unknown, it would seem quite possible that differences in the expression of this small suite of genes lie at the core of it. By identifying and characterizing the orthologous genes in *Fragaria*, it may be possible to pinpoint the location of the mutation or mutations involved in the shift from short day to everbearing or day-neutral flowering. This could in turn be the basis for molecular markers to assist in breeding, a target for genetic engineering, or even shape cultural practices by clarifying the interaction of the different physiological mechanisms. Such knowledge may have impacts even outside of strawberry, particularly for the other important crops within the Rosaceae.

To that end, efforts were made to identify the genes comprising this network in *Fragaria*, and the expression of some of these genes was examined in strawberry plants under long and short day conditions.

Results

Identification of Rosaceae Flowering Gene Homologs

Screening of *Fragaria* EST libraries developed in this laboratory (Folta et al., 2005, as well as others as outlined in Chapter 3) revealed a number of sequences related to transcripts known to be involved in flowering in *Arabidopsis*, rice, or apple (Tables 4-1 & 4-2). In addition to *Fragaria* transcripts, ESTs from other Rosaceae species were also searched for MADS-box and FT/TFL1-like genes, in order to gain insight into the nature of these genes and gene families within the larger taxon, and to help fill in gaps where no strawberry genes are known but may exist.

MADS-Box genes

Six strawberry MADS-Box genes were identified in ESTs, adding to the three already documented in GenBank. Most had fairly clear correspondence with one of the fifteen documented MADS-Box genes in apple, although not all had clear homologs in Arabidopsis. These included genes related to the Arabidopsis transcription factors *SOC1*, *SEPALLATA3, PISTILLATA, SHORT VEGETATIVE PHASE, SHATTERPROOF1/2,* and *AGAMOUS-LIKE14*, as well as one transcript not closely related to any Arabidopsis gene but somewhat similar to the apple *MdMADS11*. These were designated, in order, *FraSOC1, FrvSEP3, FrvPI, FrvSVP, FrvMADS1, FrvMADS2,* and *FrvMADS3*. These genes are listed in Table 1, and their implied relationship to the other known Rosaceae MADS-Box genes and a selection of Arabidopsis MADS-Box genes are shown in Figure 1.

FraSOC1 was first identified as a partial transcript (GenBank Accession CO817776) resembling a MADS box transcription factor among a small EST library developed from whole plant tissues of *F.* ×*ananassa* 'Strawberry Festival' (Folta et al., 2005). Because the glycerol stock of this clone was available, we were able to sequence the gene from the other end, obtaining the entire sequence. The 642 bp coding region encoded a predicted MADS Box transcription factor, with high similarity to the Arabidopsis *SOC1*, with 63% identity at the amino acid level. The closest overall match annotated in the public databases was *VvMADS8*, again from grapevine (*Vitis vinifera* L.), to which it shared 68% identity at the amino acid level. Other than this strawberry gene and the previously identified relative in apple (Valazideh et al., 2006), no apparent *SOC1* homologs were found among Rosaceae ESTs. A second *Fragaria* gene, *FrvMADS3* (GenBank Accession DY675364), also grouped within the *SOC* clade, but more closely resembled *AGL14* than *SOC1*.

FT/TFL1 genes

An EST (DY674124) bearing considerable similarity to the floral repressors *TFL1* and *ATC* was also identified among sequences derived from cold-stressed seedlings of the *F. vesca* selection Hawaii-4. This transcript was initially passed over because BLAST-X searches showed only partial similarity to known genes. Upon closer inspection, it became clear that the transcript retained 147 bp of intron sequence at a site near the middle of the gene, which resulted in a frameshift, causing multiple stop codons and corrupting more than half of the predicted protein. This likely represents only a partial failure to complete splicing, as the apple (DQ535888) and Arabidopsis (NM_120465) genes each contain three introns. With the exon sites identified by comparison to these likely homologs, the spliced transcript yielded a coding region of 666 bp and encoded a protein with 83% identity to *ARABIDOPSIS THALIANA CENTRORADIALIS* (*ATC*), and a lower 63% identity to the Arabidopsis *TFL1* (Figure 4-2), although the proportion of positive matches, 91% and 85%, respectively, were much closer. The most similar annotated transcript was from *Populus*, a "Flowering Locus T-like protein" (BAD22601), with which the protein shared 85% identity. At the nucleotide level, the most similar annotated sequence was again from *Poplar, PnTFL1* (AB181184), with an e-value of e-60, and once again this transcript was significantly more similar than the nearest Rosaceae gene, *MdTFL1-1* (AB052994), with an e-value of 9e-28.

Efforts to amplify a strawberry ortholog of *FT* from genomic or cDNA using degenerate primers based on apple and Arabidopsis sequence were unsuccessful, as was colony hybridization with Arabidopsis *FT* as a probe, and no likely candidates were identified in any of the EST libraries. No *Fragaria* sequences were identified with significant similarity to the other members of the Arabidopsis family: *TSF, MFT* (*MOTHER OF FT*), and *BFT* (*BROTHER OF FT*), however apple ESTs resembling *MFT* and *BFT* were identified (GenBank Accessions

EB134193 and EB138045, respectively), as well as a partial transcript of a *MFT*-like gene from almond (GenBank accession BU574411). Relationships of apple and strawberry amino acid sequences with members of the *FT/TFL1* family in Arabidopsis are shown in Figure 4-2.

Other genes thought to be involved in flowering

Also identified were a number of elements of the circadian clock, including apparent orthologs of several response and pseudo-response regulators, *TOC1, FKF1*, *ZTL*, as well as several genes with roles in photomorphogenesis, including genes similar to *HY5* and hemeoxygenase (Table 1). Attempts to use degenerate primers based on consensus sequence from apple, pear, and Arabidopsis *LEAFY* genes to amplify the strawberry ortholog from either cDNA library or genomic DNA were not successful. However, a fosmid from *F. vesca*, containing the genomic sequence of *LEAFY* was obtained from Dr. Thomas Davis of the University of New Hampshire.

***FrSOC1* Expression**

Surprisingly, RT-PCR assays showed *FrSOC1* expression did not parallel flowering as expected. Rather, in 'Camarosa', *FrSOC1* generally followed *FrCO* expression under short days, but with a lag of several hours, with a peak near the end of the day and tapering off into the night. Under long days, however, the expression levels in all but 'Diamante' were extremely high, and remained so through out the course of the day (Figure 4-3). In 'Diamante', the day-neutral genotype, however, *FrSOC1* expression followed similar patterns under both conditions.

***FrTFL1* and *FrLFY* Expression**

Because of the unexpected results seen with *FrSOC1,* RT-PCR assays of *FrLFY* expression were also conducted on the short day octoploid cultivar, Camarosa. Although expression levels were low, an increase to 36 cycles revealed expression of *FrLFY* (Figure 4-4) and *FrTFL1* (Figure 4-5) to be the opposite of that anticipated by the Arabidopsis model, just as

with *FrSOC1*. Under short days, when 'Camarosa' was initiating flowers, neither was detectable in leaf tissues. Under long days, when growth was primarily vegetative, however, both were expressed at all time points, though levels were low.

Discussion

Rosaceae MADS-Box Genes

MADS-box genes play critical roles in the a number of plants processes, especially in the development of fruits and flowers (Becker and Theissen, 2003), and as such are important targets of study in crop plants. The Arabidopsis MADS-box gene family comprises roughly a hundred members, though only one group, the so-called MIKC class which contains 39 Arabidopsis genes, has been functionally characterized to a significant extent (Becker and Theissen, 2003). Becker and Theissen (2003) break this class into fourteen groups, one of which, TM8, does not exist in Arabidopsis. The eight *Fragaria* MADS-box transcription factors identified in this study along with those identified by previous researchers represent seven of these groups. An eighth group, AP1-like MADS-box factors, is represented in several other species within the family, including peach (*PrpMADS6*), loquat (*EjAP1*), almond (*PrdMADS2*), and several genes in apple (*MdAP1, MdMADS5, MdMADS2, MdMADS12,* and *MdMADS12a*), but none are yet known in strawberry.

The six groups not yet attested in any of the Rosaceae are the *AGL12*, *AGL15*, *TM8*, *TT16*, *ANR1*, and *FLC* groups. Of these, *FLC* is the best characterized, playing a critical role in the regulation of flowering by non-photoperiodic factors, especially vernalization (Boss et al., 2004). It is worth noting that *FLC* orthologs have been identified only within the Brassicaceae, and are not functional within all ecotypes of Arabidopsis, nor have *AGL15* genes (Becker and Theissen, 2003). With the exception of the *ANR1* group, none of these groups were attested in the legume species Medicago, soybean, and lotus (Hecht et al., 2005). The absence of *AGL12, AGL15,* and

ANR1-type genes may be explained by the absence of root-derived Rosaceae ESTs, as these genes appear to be primarily active in root development (Becker and Theissen, 2003). The remaining groups are mostly poorly characterized in Arabidopsis.

Among the most numerous Rosaceae MADS-box genes are those of the *SEPALLATA* group. The *SEP* group in Arabidopsis consists of four members that interact with other MADS-box proteins to shape organ identity in the four whorls of flower development (Ditta et al., 2004). Two such genes were identified in strawberry, *FraMADS-RIN* (Vrebalov and Giovannoni, 2002), with greatest similarity to *AtSEP1* and *AtSEP2*, and a *F. vesca* EST we designated *FrvSEP3*. *AtSEP1* and *AtSEP2* are very similar to each other, and likely represent recent duplications, and this may account for the seven apple genes in this group, although in the case of apple, these duplications may also be the result of polyploidization in the evolution of this species'. The bulk of the Rosaceae *SEP* genes most closely resemble the *SEP1/SEP2* genes of Arabidopsis, including *FraMADS-RIN*, peach and almond genes, and all but one of the apple genes in the group. *FrvSEP3* and the pear gene *PbMADS2* (GenBank Accession AB265800) most closely resemble *SEP3*, whereas *MdMADS4* does not bear clear relation to a specific Arabidopsis member, though it most closely resembles *SEP4*. In Arabidopsis, the *SEPALLATA* genes appear to be largely redundant in function, and plants lacking all three produce flowers composed solely of sepals (Pelaz et al., 2000). Sung et al. (2000) investigated the expression of two such genes from apple, *MdMADS3* and *MdMADS4*, and found very different expression patterns, with the first expressed only in the floral primordium, whereas the second was expressed throughout floral and early fruit development, particularly in vascular bundles. Yao et al. (1999) found seven MADS box genes were expressed in distinct patterns during flower and fruit development. It seems likely that *Fragaria* genes within this family also play roles in reproductive development,

although the developmental differences between Arabidopsis, apple, and strawberry fruit require some caution in predicting function based on these other species.

One new strawberry MADS-box gene, designated *FrvMADS3*, shares some similarities with the SEP subfamily, but appears most closely related to the AGL6 group, which it shares with the apple gene *MdMADS11*. The function of these genes is poorly understood. In Arabidopsis, their expression seems to be confined to floral organs (Becker and Theissen, 2003) There is evidence that *OMADS1*, an AGL6-type gene from orchid, interacts with *OMADS3*, a factor involved in flower formation, and promotes flowering when expressed ectopically through the upregulation of *SOC1* and *FT* in Arabidopsis (Hsu et al., 2003). Similar results were achieved by Tian et al. (2005) in their study of another AGL6-like gene, *DlMADS18*, from bamboo (*Dendrocalamus latiflorus* Munro).

The AP3 and PISTILLATA class genes are class B organ identity genes, and combine with class A and C genes to specify petals and stamens, respectively (Krizek and Meyerowitz, 1996). Both have been previously identified in apple (Yao et al., 2001) and rose (Kitahara et al., 2001). The three apple mutants—'Rae Ime', 'Spencer Seedless', and 'Wellington Bloomless'—which produce apetalous flowers and parthenocarpic fruit, were found by Yao et al. (2001) to contain retrotransposon insertions in introns of *MdPI,* abolishing expression and suggesting a critical role for the gene in both fruit and flower development. The strawberry *PI* gene identified here might thus be an interesting target for RNAi- or antisense-based suppression of expression. Parthenocarpy might be a means to limit malformation of fruit, which can result from incomplete pollination, and might also increase fruit size.

The gene designated *FrvSVP* most closely resembles the SVP group of MADS-Box genes. This group appears to play a role in the control flowering in Arabidopsis, with both *SVP*

(Hartmann et al., 2000) and *AGL24* (Yu et al., 2002) acting as repressors of flowering. *SVP*-like genes have been identified in model legumes (Hecht et al., 2005) and in apple and peach. A peach gene, closely resembling the strawberry *FrvSVP* (D. Bielenberg, personal communication), has been identified as among the genes deleted in the *evergrowing* mutation in peach (Bielenberg et al. 2004). The apple gene, *MdJOINTLESS* (Heo et al., 2006), differs significantly from those of strawberry and peach, more closely resembling the tomato gene of the same name.

The final major group are the *SOC1* like genes. The role in flowering of *SOC1*, the best known of these genes, has been described earlier in this work. Related genes in apple (Mahna et al., 2006), rice (Lee et al., 2004), eucalyptus (*Eucalyptus grandis* Hill ex. Maiden) (Watson and Brill, 2004) and aspen (Cseke et al., 2003) have been identified and demonstrate evidence of similar functions. *VvMADS8*, from grapevine, to which *FraSOC1* is most similar, has recently been demonstrated to hasten flowering in transgenic Arabidopsis, supporting the idea that it may be that species equivalent of *SOC1* (Sreekantan and Thomas, 2006). In some cases, such as eucalyptus (Watson and Brill, 2004) and pea (Hecht et al., 2005), there are multiple family members that appear to fill the role of *SOC1*. The other members of the *SOC1* subfamily in Arabidopsis are not well-characterized, but at least two, *AGL14* and *AGL19*, are expressed solely in roots (Rounsley et al., 1995; Becker and Theissen, 2003). *FrvMADS2*, identified among *F. vesca* seedling ESTs, is roughly equal in similarity to both of these genes, and represents the first close relative identified in the Rosaceae.

The FT/TFL1 Gene Family in Rosaceae

The FT/TFL1 gene family, as defined in Arabidopsis, *Antirrhinum*, and tobacco, is comprised of two clades, the *FT*-like genes and the *TFL1*-like genes, as well as *MOTHER OF FT* (*MFT*) which is more distantly related to both (Kobayashi et al., 1999). The *TFL1* clade is further

divided into the *TFL1*-like and *CEN*-like groups. The sole *TFL1*-like gene identified in strawberry, *FrTFL1*, most closely resembles the gene *Arabidopsis Thaliana Centroradialis* (*ATC*), a member of the *CEN* group. Although both are very similar genes in both structure and apparent function, *ATC* and *TFL1* differ primarily in the timing of their expression, as *ATC* is confined to young seedlings while *TFL1* is present in mature Arabidopsis plants (Mimida et al., 2001), and as a result, only *TFL1* plays a role in governing the reproductive transition. Despite the similarity to *ATC*, a decision was made to designate this gene *FrTFL1*, following the convention of Carmona et al. (2007). Although *FT*, *ATC,* and *TFL1* are members of the same gene family (Kobayashi et al., 1999; Mimida et al., 2001), the annotation of the *Populus* ortholog as "FT-like" is probably something of a mis-annotation, since the e-value when compared to Arabidopsis *FT* was only 1e-50, and 5e-48 when compared to *TWIN SISTER OF FT* (*TSF*), and all BLAST-X matches higher than the Arabidopsis TFL1 except the *Populus* gene were annotated as either "TFL1-like" or "CEN-like" (*CEN* being the *TFL1* homolog in *Antirrhinum* (Pnueli et al., 1998)) .

TFL1 orthologs are well-documented in the Rosaceae, though the work is confined to the Maloideae. Consistent with the polyploid origins of this subfamily, Esumi et al. (2005) identified two closely related *TFL1* genes in each of six maloid fruit tree species. These genes were expressed in the flower buds of all six species, and elsewhere in few of the species, including in the peduncles of pears and the stamens of apples. Most studies of *TFL1* in Rosaceae have focused on its possible role in maintaining juvenility in fruit trees, an important factor given the long delay before fruiting seen in many species. Kotoda and Wada (2005) found a clear relationship between the juvenile state and expression of *MdTFL1*, and, by using an antisense

construct with this gene, were able to significantly shorten the juvenile period in transgenic 'Orin' apples (Kotoda et al., 2006).

Juvenility is not a major concern in strawberries, but *AtTFL1*'s role as a determinant of meristem identity may suggest a possible function for *FrTFL1* in determining switch between vegetative growth and terminal inflorescence in strawberry. In Arabidopsis, *tfl1* mutants flower quickly and terminate inflorescence growth immediately in a single terminal flower whereas 35S::*TFL1* constituitive overexpression lines had extremely long vegetative phases and many axillary buds that would normally have terminated in flowers instead formed long shoots lacking cauline leaves (Radcliffe et al., 1998). Such a transition from terminal inflorescence to elongated axillary shoot seems reminiscent of the switch from flowering to runnering observed in strawberry, and it would seem possible that homologs of *TFL1* might be involved in the process. It is worth noting that the subgroup involved in inflorescence develoment, either *CEN-like* or *TFL1-like*, varies among species, with the *CEN* genes filling these roles in *Antirrhinum*, tomato, and possibly tobacco, prompting Mimida et al. (2001) to speculate that while the two subgroups diverged before the origination of the angiosperms, it was not until later that one or the other group was "recruited" to participate in inflorescence development. This difference exists solely through the expression of the genes, not through their function, as *ATC* has been shown to complement *tfl1* loss-of-function mutants and vice versa (Mimida et al., 2001). Although the original transcript was obtained from seedling tissues, the RT-PCR results show it to be expressed to a limited extent in mature leaf tisses as well, so it is possible that in *Fragaria* it is the *CEN*-like group which is involved in inflorescence development. However, preliminary work reported by Esumi et al. (2006) suggests that in pear and quince trees, expression of *TFL1* in the meristem correlates with the development of simple versus complex inflorescences, and a more

TFL1-like gene, rather than the *CEN*-like one described in this study, may be controlling such development in *Fragaria*.

Despite many efforts, no *FT* sequences were obtained from strawberry species. An apple homolog of *FT* has previously been characterized (Kotoda and Wada, 2005), and several orthologs of *FT* other related genes have been identified among apple, peach, and almond ESTs (although the peach *FT* mapped by Silva et al., 2005 more closely resembles *SEP1*), so it seems unlikely that the family would be completely unrepresented in strawberry. More likely the issue is simply one of very low levels of transcript and a collection of EST data that is still relatively small compared to other species (as of March 3, 2007. there are 19,091 *Fragaria* ESTs, compared to 91,745 for *Prunus*, and 260,927 for *Malus*). Only two *FT* transcripts could be located among apple ESTs, so it is not surprising that it has not appeared in less than a tenth as many strawberry sequences.

The *FT* subfamily in apple is also represented by orthologs of *MFT* and *BFT*. Neither gene is well-characterized in Arabidopsis, but *MFT* has been demonstrated to accelerate flowering, and may serve a role similar to that of *FT,* albeit at a lower level, whereas overexpression of *BFT* has no apparent effect on flowering (Yoo et al., 2004). It is interesting that these two genes, apparently of relatively minor importance in *Arabidopsis*, are preserved in apple (and *MFT,* at least, in almond), indicating that they are both ancient and persistent. Yoo et al. (2004) suggest that differences in *MFT* expression between Arabidopsis ecotypes may imply an adaptive role, despite a largely redundant function. The fact that it is retained in a relatively distantly related family may support this idea.

Other Flowering Genes

A number of components of the circadian clock in strawberry were also identified. Defects in circadian function have been shown to alter flowering time in a number of species (Schaffer et al., 1998; Yamamoto et al., 2003; Zhao et al., 2005), and the day-neutral trait in barley has been shown to be the result of a mutation in *Ppd-H1*, a pseudo-response regulator that is part of the circadian clock (Turner et al., 2005) Several of these response and pseudo-response regulators were identified, including one, a relative of *ARR9*, that was mapped in the FVxFN diploid reference population (Sargent et al., 2005). However, the location, at the top of linkage group II, did not correspond to either of the two QTLs for flowering time known in that population, (Sargent et al., 2006). A pseudo-response regulator similar to the Arabidopsis *APRR7*, ortholog of *Ppd-H1*, was also identified among *F. vesca* ESTs.

Very few genes related to photoreceptors were identified among the ESTs or by conserved primers. Although hemeoxygenase is involved in the phytochrome chromophore biosynthesis (Muramoto et al., 1999), no phytochrome or cryptochrome genes were identified, nor were other flowering-related photoreceptors with the exception of *FKF1*. Thus, it remains somewhat difficult to understand the dynamics that underlie the unusual reaction of strawberry plants to red and far-red light demonstrated by Vince-Prue and Guttridge (1973) and Kadman-Zahavi and Ephrat (1974) and outlined in the previous chapter. Although careful spectral studies, such as these, can determine the gross physiology of these interactions, sequence data would allow more targeted studies of the mechanics at work.

***FrSOC1*, *FrLFY*, and *FrTFL1* Expression**

The results of the RT-PCR studies are perhaps the most surprising of this project, and would seem difficult to adequately explain without additional information. It was anticipated that expression of *FrSOC1* and *FrLFY* would parallel the occurence of flowering, as their orthologs

in Arabidopsis (Samach, 2000) do so. In both cases, expression of both genes are upregulated under photoperiods conducive to flowering: long days in Arabidopsis and short days in rice. There is evidence that *SOC1* acts at least in part by upregulating *LFY* (Moon et al., 2005), so it is not surprising that the two genes show similar expression patterns, though no *FrLFY* transcript is in evidence under short days, despite significant *FrSOC1* expression. The rice ortholog of *LFY*, *RFL,* does not seem to directly promote flowering itself, and instead seems to be down-regulated in flowering plants. Overexpressing this gene in rice inhibits flowering, unlike *AtLFY*, which promotes the conversion of floral meristems into flowers (Kyozuka et al., 1998).

Because no *Fragaria* equivalent to *FT* could be identified, we are only able to see part of the floral induction network model described in Arabidopsis. It is possible that the primary stimulus of flowering comes through that part of the pathway, as *FT* has been shown to be able to trigger flowering through genes other than *SOC1* and *LFY* (Yoo et al., 2005). What is perhaps more puzzling is the fact that upregulation of the other two floral integrators, *SOC1* and *LFY*, appear *not* to trigger flowering, but might correlate better with runnering.

Although the *FrTFL1* data is also the reverse of what was anticipated, the extremely low amounts seen may not be meaningful levels of expression. The rarity of this transcripts suggests that it may localized elsewhere in the plant, and that this may indeed be a homolog of *CEN*, rather than *TFL1*, and thus may be expressed primarily during early hypocotyl development (Mimida et al., 2001).

Another reason these results are unexpected is the relationship they bear to *FrCO* expression. *CO* and its orthologs have been shown to play either of two roles. In Arabidopsis and other long day plants, it serves as a promoter of flowering by upregulating *FT, SOC1*, and, indirectly, *LFY* (Samach, 2000), but in rice and many other short day plants, however, it

functions to suppress expression of these same genes (Kojima et al., 2002). In the previous chapter, it appeared that because high levels of *FrCO* were only present during short days, when strawberry was flowering, that it must function as a promoter of flowering. The expression of *FrSOC1* under short days, which appears to roughly track expression of *FrCO*, would seem consistent with that analysis, if not for the fact that expression is considerably higher under the non-inductive long day conditions. Nor does *LFY*, a component of the Arabidopsis network downstream from *SOC1*, appear to be activated at all by high levels of *FrCO* expression. This would suggest one of three possibilities: either control of flowering is dependent on other integrators, perhaps *FT*, that the floral stimulus conveyed by *FrSOC1* and *FrLFY* is being gated further downstream along the pathway, or that the effects of *FrSOC1* and *FrLFY* differ from those of their Arabidopsis counterparts.

In apple, another Rosaceae species, two *LFY* homologs have been identified: *AFL1* and *AFL2*. These two genes have been shown to promote flowering when overexpressed in transgenic Arabidopsis, as does the native *LFY* (Wada et al., 2002). However, their overexpression in transgenic apple plants does not accelerate flowering (Kotoda et al., 2006), suggesting that while they are expressed in floral buds, perhaps *LFY*-type genes do not necessarily function as floral integrators in that species. Because *LFY* plays another role as a regulator of floral organ differentiation (Simon et al., 1996), it is possible that the gene has retained only this function in the Rosaceae, while losing its role as an integrator of floral signalling, which might explain why the gene has been preserved and expressed. No such other role is known for *SOC1*, although complex interactions are not unknown among MADS-box genes.

Apple, however, appears to be a day-neutral species (Carew and Battey, 2005), at least in regards to flowering. Although photoperiod seems to play a role in controlling dormancy in *Prunus* species (Besford et al., 1996), there appears to be little evidence of photoperiod sensitivity in regards to flowering in *Prunus* species, although there is some suggestion that flowering in at least peach and sand cherry (*P. besseyi* L.H. Bailey) is inhibited by short photoperiods (Aerov, 1963). Clear short-day photoperiodic initiation of flowering in the Rosaceae would seem to be limited to the Rosoideae subfamily, as seen in strawberry, biennial-fruiting raspberry (Williams, 1959, 1960), and the cinquefoil species *Potentilla glandulosa* and *P. fruticosa* (Ahmadi et al., 1991), although some members, such as roses, appear to be normally day-neutral (Zieslin and Mor, 1990). This raises the possibility that photoperiodic flowering in the Rosaceae may have been lost during the course of evolution, and re-emerged in one branch of the family through a novel mechanism. This would explain the striking differences with previously documented systems in other species. This might also explain unsual spectral sensitivities as well (Vince-Prue and Guttridge, 1973). The differences in the photoperiod pathway between Arabidopsis and rice demonstrate how a similar set of parts can be re-tooled by evolutionary forces to produce a very different result, and it seems likely that any novel mechanism would make use of many of the existing elements of the pathway, though perhaps in somewhat modified roles.

As with the *FrCO* portion of this study, one aspect in which our understanding is lacking is at the protein level. High transcript levels do not necessarily mean high levels of protein, nor do low transcript levels necessarily mean low levels of protein, as many factors have been shown to affect the stability and persistence of proteins (Valverde et al., 2004). It may be that while expression of *FrSOC1* and *FrLFY* transcripts are high, the resulting proteins are being rapidly

degraded or otherwise prevented from functioning. However, it must be noted that no such post-translational regulatory mechanisms involving these particular genes appear to have been previously reported in other species.

Methods and Materials

Identification of Rosaceae Flowering Genes

Predicted amino acid sequences of Arabidopsis genes were queried against GenBank EST databases via the BLAST-X algorithm (Altschul et al. 1997), with the output limited to the Rosaceae. These genes included *FT*, *TSF, MFT, BFT, TFL1, TFL2, ATC, PFT1, FKF1, ZTL, TOC1, CCA, LHY, PHYA, PHYB, PHYC, PHYD, PHYE, CRY1, CRY2, LFY, SOC1,* the MADS-Box domains of *AP1*, *AG,* and *SEP3,* and the CCT domains of *CO* and *TOC1*. Additionally, the twenty published apple MADS-box gene nucleotide sequences (*MdMADS1-14, MdPI, MdJOINTLESS, MdSOC1, MdAG, MdAP1*, and *MdTM6*) were also queried against Rosaceae EST sequences, using the BLAST-N protocol (Altschul et al., 1997), as were the apple genes *AFL1, AFL2, MdTFL1-1, MdTFL1-2,* and *MdFT*. Some *Fragaria* flowering genes, including heme-oxygenase, *FraHY5*, and *FraSOC1*, were noted by visual inspection of BLAST-X results lists prepared during analysis of a small *F.* ×*ananassa* library (Folta et al., 2005).

Phylogenetic Tree Construction

Trees were constructed based on full-length amino acid sequence using the "Neighbor Joining" (NJ) method of Saitou and Nei (1987), through the CLUSTAL-W alignment program (online at http://www.ebi.ac.uk/clustalw/) (Higgins et al., 1994). The resulting DND data file was saved and exported to TreeEdit 1.0a10 Carbon for MacOS X, which was used to draw the trees.

RNA Extraction and RT-PCR Experiments

RNA was extracted from mature leaf tissue using a modification of Chang et al. (1993), as described in Folta et al. (2005) and in Chapter 3. RT-PCR experiments were conducted as

described for the second *FrCO/FrCOL* expression experiment in Chapter 3, with the exception of *FrLFY*, for which 35 cycles of PCR were used.

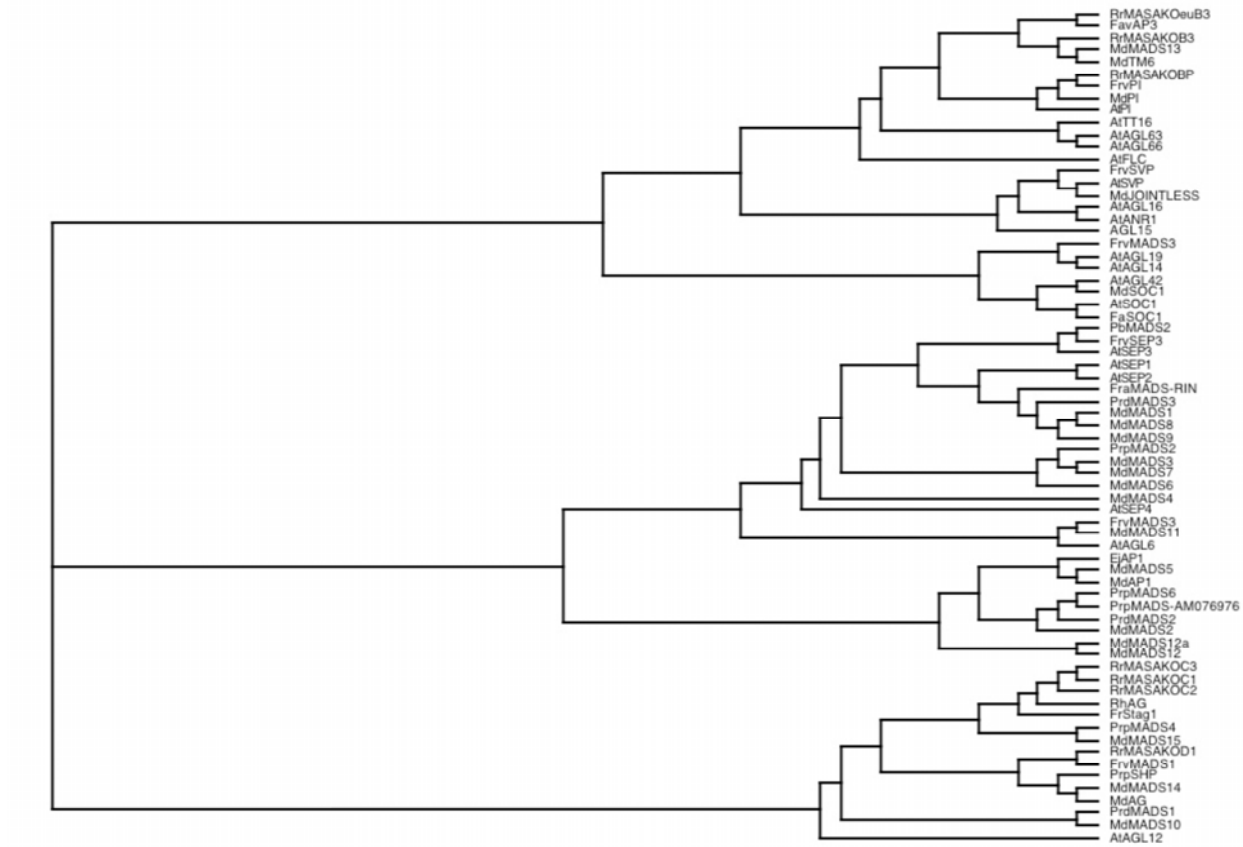

Figure 4-1. NJ tree showing similarity between full-length amino acid sequences of Rosaceae and Arabidopsis MADS-Box genes. Md is *Malus x domestica*, Prp is *Prunus persica*, Prd is *Prunus dulcis*, Pyb is *Pyrus x bretschneiri*, Rr is *Rosa rugosa*, Rh is *Rosa hybrida*, Ej is *Eriobotrya japonica*, and Frv and Fra are *F. vesca* and *F. ×ananassa*, respectively.

Table 4-1. Identified *Fragaria* genes related to flowering and photoperiodic response, with the closest match at the amino acid level among Arabidopsis and all annotated transcripts.

Strawberry Gene	Arabidopsis Match	E Value	Closest Match	E Value
FrvTFL	ATC	6e-73	Flowering Locus T-like protein (*Populus nigra*)	6e-83
FraSOC1	SOC1	1e-45	VvMADS8 (*Vitis vinifera*)	1e-54
FrvLFY	LFY	9e-103	LFY-2 (*Cydonia oblonga*)	2e-123
FrvFKF1	FKF1	7e-42	FKF1 (*Arabidopsis thaliana*)	7e-42
FrvPI	PI	7e-55	MASAKO BP (*Rosa rugosa*)	2e-87
FrvSVP	SVP	9e-38	MADS1 (*Populus tomentosa*)	5e-43
FrvAGL14	AGL14	2e-52	VvMADS8 (*Vitis vinifera*)	1e-53
FrvSEP3	SEP3	2e-90	PbMADS2 (*Pyrus* x*bretschneideri*)	5e-113
FrvMADS11	AGL6	5e-72	VvMADS3 (*Vitis vinifera*)	2e-94
FrvMADS14	AGL1/SHP1	5e-80	MASAKO D1 (*Rosa rugosa*)	1e-115
FraHY5	HY5	4e-47	HY5 (*Arabidopsis thaliana*)	4e-47
FrvTOC1	TOC1	5e-70	TOC1 (*Arabidopsis thaliana*)	5e-70
FraRR9/FrvRR9	ARR9	3e-31	Response regulator receiver (*Medicago trunculata*)	93-35
FrvPRR5	APRR5	1e-56	APRR5 (*Arabidopsis thaliana*)	1e-56
FrvPRR7	APRR7	8e-81	APRR7 (*Arabidopsis thaliana*)	8e-81
FrvZTL	ZTL	1e-95	Spercat/scavenger receptor; cylin-like F-box; galactose oxidase, central (*Medicago trunculata*)	4e-104

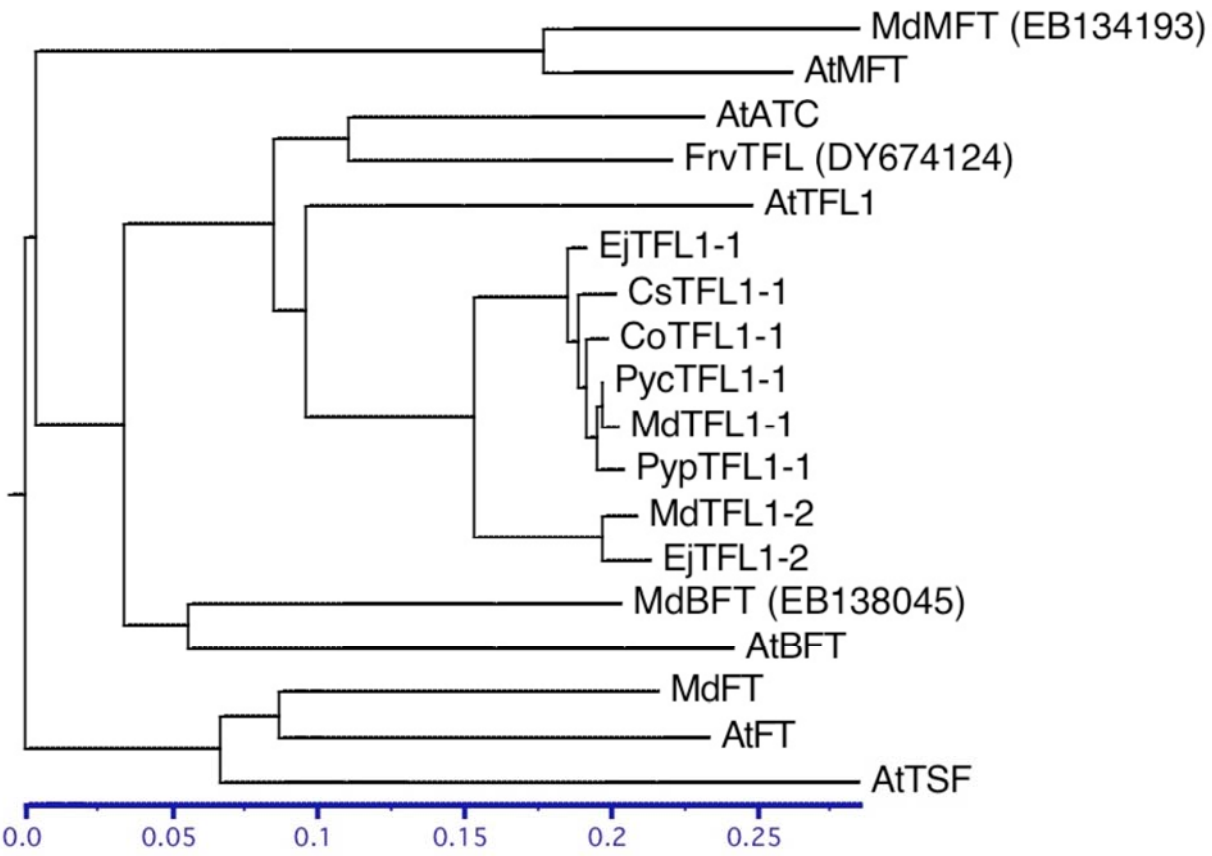

Figure 4-2. NJ phylogeny tree showing relationships between Arabidopsis and Rosaceae members of the FT/TFL1 gene family, based on full predicted protein sequence.

Table 4-2. RT-PCR primers for strawberry flowering genes and controls, sequence source, T_M, and approximate size in cDNA (as calculated from sequence).

Gene	Primers	Sequence	TM (°C)	EST	bp
Flowering Genes					
FrSOC1	RN-FrSOC1-F	cgcaagctcgagcatgcagg	61.8	CO817776	481
	RN-FrSOC1-R	aagctagtgcttcgatctcc	54.2		
FrLFY	FvLFYintron2-F	agcgtggtgaaaaatgtcccacc	60.3	—[z]	409
	FvLFY-R	tcagtagggcagctgatgagc	59.1		
FrTFL1	RN-TFL1-F2	agcttgcagcttcagaaagagc	58.1	DY674124	(246)[y]
	RN-TFL1-R	tggcacgtacaagtgcttccaa	59.4		403
Control Genes					
FrActin1	RN-Actin-F	tggctgtgcacgatgattgc	58.8	DV439971	430
	RN-Actin-R	taacttcccaccagatatcc	50.9		
FrUbiq1	RN-Ubiq-F	aaccaaccgtccaacaatcccaac	60.1	CX661133	414
	RN-Ubiq-R	accggatcagcagaggttgatctt	60.0		

[z] Sequence obtained from fosmid sequenced by Tom Davis, U.N.H.

[y] Predicted size of transcripts when fully spliced

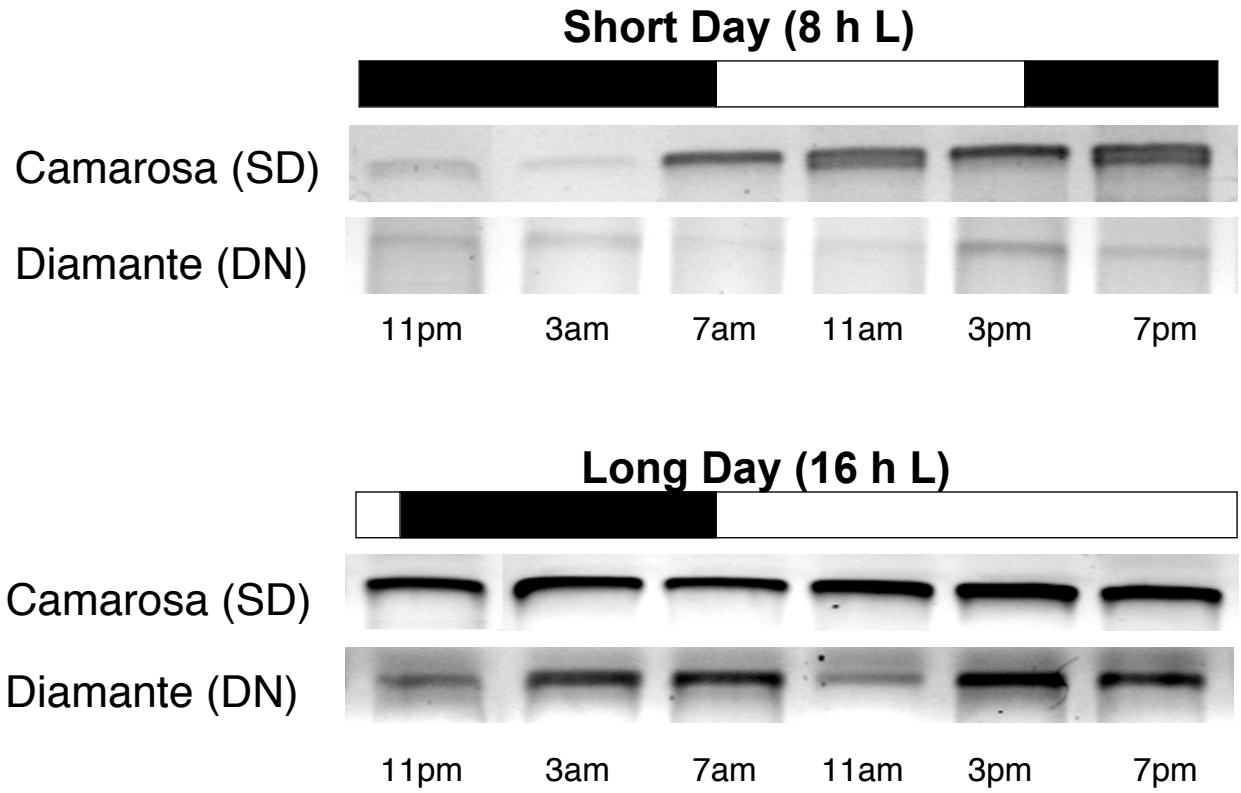

Figure 4-3. RT-PCR assay of expression of *FrSOC1* under short (8 h) and long (16 h) photoperiods in 'Camarosa' (SD, octoploid) and 'Diamante' (DN, octoploid).

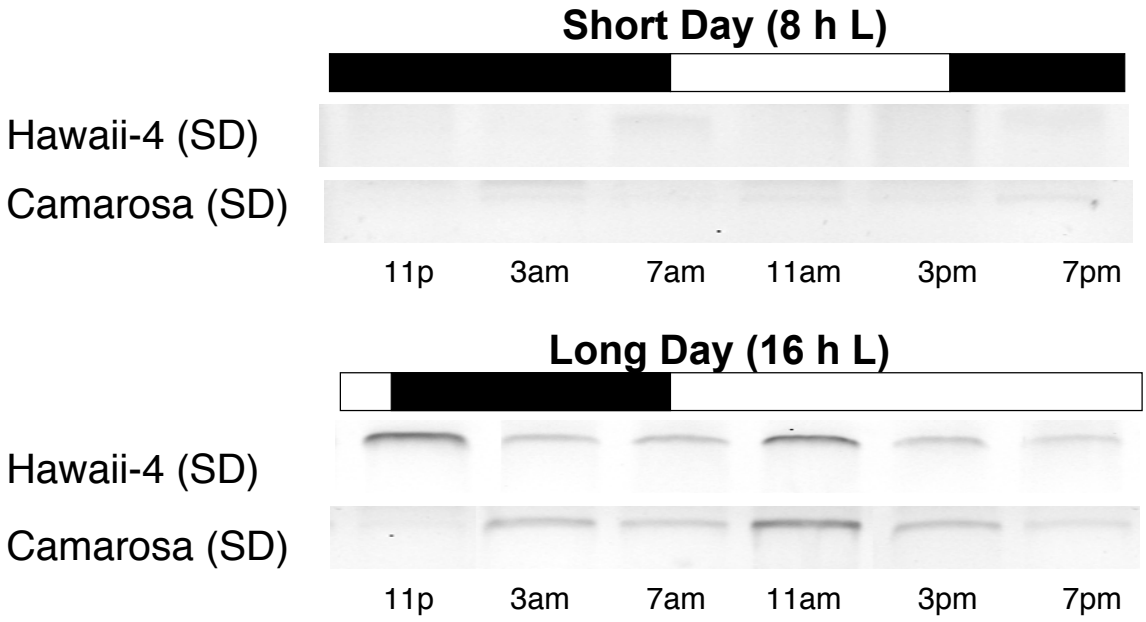

Figure 4-4. RT-PCR assay of expression of *FrLFY* under short (8 h) and long (16 h) photoperiods in Hawaii-4 (SD, diploid) and 'Camarosa' (SD, octoploid).

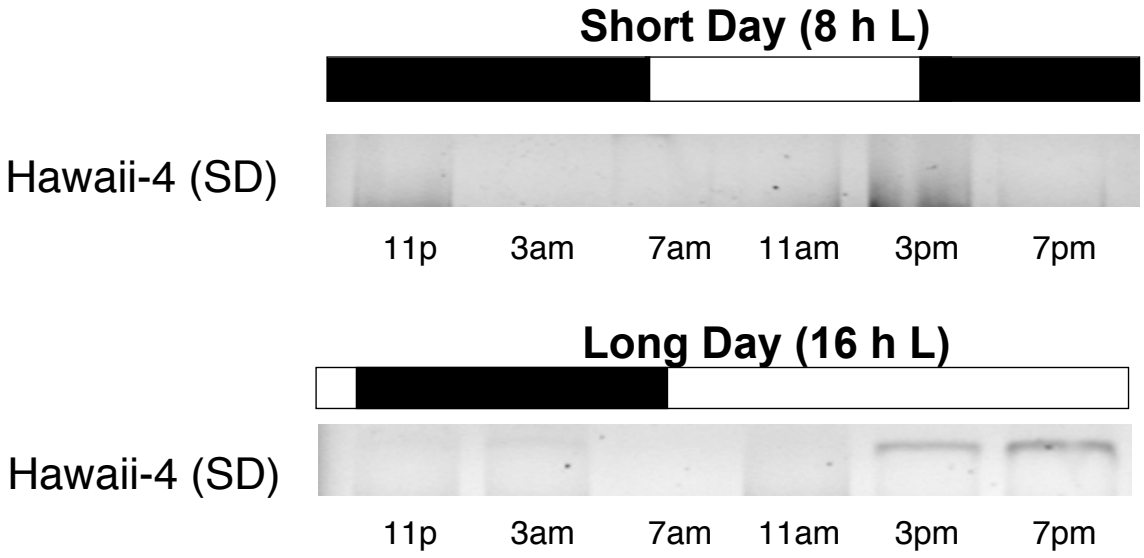

Figure 4-5. RT-PCR assay of expression of *FrTFL1* under short (8 h) and long (16 h) photperiods in Hawaii-4 (SD, diploid).

CHAPTER 5
Fragaria ALLELES OF POLYGALACTURONASE INHIBITOR PROTEIN GENES

Introduction

At any given moment, a plant's environment is full of potentially pathogenic organisms. The fact that the vast majority of these fail to result in disease is a testament to the effectiveness of plants' many-faceted defensive mechanisms. Yet these dangers represent diverse and rapidly evolving threats to a plant survival. Furthermore, pest species are undergoing continual selection for their ability to best exploit the broad range of hosts available in the environment (Thrall and Burdon, 2003). Similarly, plants in the wild are undergoing natural selection for their ability to recognize and counter this changing threat (Stotz et al., 2000). In cultivated plants, this need for selection falls instead on the plant breeder and the farmer.

This interaction is reflected in the "gene-for-gene" theory of resistance, a dominant paradigm in plant pathology for nearly half a century. This theory, first developed by Flor (1956), through his work on rust resistance in flax, proposes that for each resistance gene there is a matching avirulence gene in the pathogen with which it interacts. This has been further refined into a receptor-ligand model, in which each resistance gene encodes a receptor that recognizes the products of avirulence genes in the pathogen (van der Biezen and Jones, 1998).

Cell wall-degrading enzymes are used by pathogens to penetrate plant tissue and release nutrients, and among these are the endopolygalacturonases (PGs). These proteins have been shown to be a primary cause of damage caused by several insect species (Girard and Jouanin, 1999; Boyd et al., 2002), fungal pathogens (Kahmann and Basse, 2001), bacteria (Oeser et al., 2002) and possibly nematodes (Jaubert et al., 2002). Pathogens often produce a spectrum of PGs, to allow pathogenesis in various hosts and under diverse conditions (De Lorenzo et al., 2001),

and PGs from different isolates or races of pathogens frequently exhibit polymorphism (Caprari et al., 1993; Daroda et al., 2001; Poinssot et al., 2003).

Plants combat these effects in part through polygalacturonase-inhibiting-proteins (PGIPs) (De Lorenzo and Ferrari, 2002). PGIPs not only counteract the ability of PGs to break down cell walls, but also seem to be capable of inducing defense responses (De Lorenzo et al., 2001) by encouraging the formation of long-chain oligogalacturonides. PGIPs are up-regulated in response to stress, wounding, and pathogen attack (De Lorenzo et al., 2001; Yao et al., 1999), and overexpression of PGIPs in transgenic plants have been shown to inhibit fungal colonization (Powell et al., 2000; Ferrari et al., 2003).

PGIP proteins possess a Leucine-Rich Repeat (LRR) region, shared by many resistance (*R*) gene products (Martin et al., 2003) and other defense components. In PGIPs, this region is generally ten repeats long, and is the site of much of the diversity in the family. A single amino acid variation in this region has been shown to confer the ability to recognize a specific PG (Leckie et al., 1999)

In Arabidopsis, PGIPs are encoded by a pair of adjacent, very similar genes (Ferrari et al., 2003), whereas in bean the family consists of two tandemly-duplicated pairs (D'Ovidio et al., 2004). Mehli et al. (2004) concluded that PGIP genes comprise a small family, of perhaps three to five genes, in the diploid *F. vesca* genome, as in raspberry (Ramanathan et al., 1997) and apple (Yao et al., 1999). Such gene duplications and divergence within the family may be the result of selection through coevolution with PGs from pathogens. Both Arabidopsis and bean PGIPs have been shown to differ markedly in their abilities to inhibit specific PGs, and in their expression patterns (Ferrari et al., 2003; D'Ovidio et al., 2004).

Mehli et al. (2004) cloned and characterized a *F.* ×*ananassa* PGIP gene, later designated FaPGIP1a. They found it to be highly expressed in red fruit, though there was at least a low level of expression in all other tissues examined. It also appeared to be strongly induced by inoculation with *Botrytis cinerea*, the causal agent of grey mold in strawberry. The five European cultivars used in the study showed varying base levels of expression as well as levels of induction in response to inoculation, although there was not a clear correlation between level of PGIP expression and level of resistance. Later work showed that PGIP was expressed robustly within 8 h of inoculation, and to peak at 16 h after inoculation (Mehli et al., 2005).

Schaart et al. (2005) presented a continuation of this study, identifying six distinct alleles of *Fragaria* PGIP, which they grouped as FaPGIP1, FaPGIP2, and FaPGIP3. All had similar nucleotide and amino acid sequences, and FaPGIP2 and FaPGIP3 appeared similar to *F. vesca* and *F. iinumae*, respectively, both proposed progenitor species of the octoploid cultivar (Folta and Davis, 2006). Five cultivars were tested for the presence or absence of each of these alleles, with at least two alleles identified in each cultivar. Pyrosequencing identified clear tissue-specific and allele-specific differences in expression, with a majority of expression in the leaf being PGIP2, whereas in the fruit, PGIP1 was the predominant transcript in uninoculated fruit, particularly early in development. In inoculated fruit, however, all three PGIP classes were expressed, in proportion with their representation in genomic DNA (Schaart et al., 2005)

PGIPs possess a number of characteristics that suggest they may be a worthwhile target of efforts to develop markers for resistance or to develop resistant transgenic lines. These include the fact that their expression has been shown to be induced by fruit inoculation (Mehli et al., 2004), that their effectiveness has been can be readily altered by small allelic variations, and that bean PGIP proteins have been shown to inhibit the growth of such strawberry pathogens as

Colletotrichum acutatum and *B. cinerea* (D'Ovidio et al., 2004). The family of PGIP genes in the octoploid also provides an opportunity for comparison with those of diploid species and may cast light on the origins of the octoploid species and on changes in the genome that may have occurred since those species emerged. To explore either of these aspects, it is important to have as complete an accounting as possible of the PGIP genes present in the cultivated strawberry. This report catalogs a suite of Fragaria PGIP genes and illuminates potential utility in disease resistance, as well as their use as beacons for the rapid evolution of the Fragaria genome.

Results

Identification of *Fragaria* PGIP Alleles

Among the *F.* ×*ananassa* clones, 12 unique PGIP alleles were identified, comprising the three groups defined by Schaart et al. (2005) plus a likely fourth group, that was designated FaPGIP4. Two new alleles of FaPGIP2, designated d and e, and one each of FaPGIP1 and FaPGIP3, designated d and b, respectively, were identified (Table 5-1).

Despite extensive sequencing, only one clone of FaPGIP1 was identified from products of the PGIP-seq1 and PGIP-seq2 primers, FaPGIP1d from 'Treasure'. Because of this, a second forward primer specific to FaPGIP1 sequence (FaPGIP1-F) was used with the same reverse primer and amplified an appropriately-sized fragment in all octoploid and diploid genotypes, suggesting that polymorphism might exist in the portion of sequence matched by PGIP-seq1. Clones generated by this pair from 'Strawberry Festival' and 'Treasure' were sequenced. Only FaPGIP1b-type clones were found among three sequenced from 'Strawberry Festival', however it is possible that these differed from FaPGIP1b at the 5' end, because this area was omitted with the second primer pair, and no FaPGIP1 were amplified from 'Strawberry Festival' using the original set. From 'Treasure', the three clones sequenced were either FaPGIP1b, or a previously undocumented allele, FaPGIP1d.

PCR using 'Strawberry Festival' cDNA library as template unexpectedly yielded two bands, one of the predicted size based on the mRNA sequence, and another larger fragment of approximately equal abundance, consistent with the genomic sequence. When sequenced this proved to be FaPGIP1a, an apparently unspliced transcript with the intron retained.

Although FaPGIP3 type sequences were identified in all the octoploid cultivars in this study except 'Treasure', none contained the specific allele previously identified by Schaart et al. (2005). Rather, these cultivars all contain another somewhat different allele, designated FaPGIP3b, which is completely identical to that of *F. iinumae* within the sequenced region. Such exact correspondence between *F. iinumae* and an octoploid allele lends additional evidence to the case for *F. iinumae* as an ancestor of the octoploids.

A novel allele type, FaPGIP4, was also identified in 'Treasure' and 'Winter Dawn'. This sequence, though quite similar to the others, did not appear to fit the criteria for any of the established classes in the octoploid, and appears to be more widely diverged from the rest of the family than other alleles. Much of this variation occurs in the coding region, suggesting possible functional differences. Although predicted, functional assessment of these novel alleles remains to be proven, and represents an excellent target for transgenic analysis.

Although FaPGIP1-specific primers amplified a product from the diploid species, none were sequenced. Among the clones generated by the original primers, each diploid species yielded only a single type of PGIP allele. The allele from *F. vesca* 'Pawtuckaway' was identical to FaPGIP2c, but did not exactly match any of the three GenBank accessions. However, it most closely resembled the *F. vesca* A6 allele, differing by a single nucleotide substitution, whereas that sequenced from *F. iinumae* FRA377 was identical to that in GenBank. PGIPs from *F. mandshurica* FME and *F. bucharica* FRA520 were most similar to those of *F. vesca* and the

PGIP2-type octoploid alleles, though both possessed single nucleotide polymorphisms (SNPs) distinguishing these genes from others.

Amino Acid Similarities Among Alleles

A majority of the polymorphisms identified did not result in differences in predicted amino acid sequence, and even the most dissimilar, FaPGIP4 and FaPGIP1 or FaPGIP3, shared 90% identity at the amino acid level (Table 5-2). Several of the alleles differed only in intron sequence and yielded identical predicted proteins. Proteins differed at 21 sites within the sequenced region, but fully non-synonymous substitutions occur at only four of these sites, at amino acids 36, 42, 121, and 129, along with an inserted alanine at position 61 in FaPGIP4 and and a deleted threonine in *F. mandshurica* FME (Figure 5-1). Within the characterized region, the most similar protein from other species was RiPGIP1, from red raspberry (Ramanathan et al., 1997), ranging from 82% (FaPGIP4) to 90% (PGIP2a/b/e, PGIP3a/b, and Pawtuckaway) amino acid identity.

Discussion

All the genes sequenced were remarkably well conserved. However, because changes in pathogen specificity can occur as a result of a single amino acid difference, it is possible that the sequenced alleles differ in their effectiveness in inhibiting specific pathogen PGs. Most of the variation occurs in the LRR region, which, as the putative site of specificity, is likely to be the portion of the gene under the greatest selective pressure.

Most substitutions occurring through natural variation will likely be either neutral or deleterious, with adaptive changes being comparatively rare. In genes undergoing positive selection, the ratio of non-synonymous to synonymous substitutions is generally higher than one, whereas in those undergoing purifying selection or neutral variation the number should be less, often much less. This measure is considered a stringent test of adaptive sequence evolution, but

may not identify cases of weak selective adaptation (Li, 1993).This ratio within the *Fragaria* PGIPs is slightly greater than one, and is likely indicative of adaptive change occurring within the gene family.

Stotz et al. (2000) identified nine amino acid residues in dicot PGIPs for which there was statistical evidence of positive selection, noting that a disproportionate number, though not all, fell into the xxLxLxx beta-sheet regions. Only one of these residues, Ala207 (position based on PvPGIP1 sequence), corresponds to the position of a non-synonymous substitution within the *Fragaria* PGIPs. FaPGIP1d has an asparagine in this position, whereas all others have aspartic acid. Another non-synonymous substitution, a serine rather than an arginine at position 104 in FaPGIP3a, also occurs within a beta-sheet region.

Leckie et al. (1999) identified a particular amino acid in bean PGIP, located at 253 in PvPGIP2, as being critical to the ability to inhibit PG from *Fusarium moniliforme*. In bean, this residue was either a Q or a K, with isoforms possessing the Q capable of recognizing FmPG, and those with the K incapable. Switching this residue to a Q conferred the ability to recognize FmPG on those proteins that were originally incapable, albeit at a relatively low efficiency. Although the strawberry PGIPs differ considerably from their counterparts in *P. vulgaris*, with the most similar pair, *F. mandshurica* PGIP and PvbPGIP2, sharing only 49% amino acid identity, it is interesting to note that all contain a conserved Q residue in this location.

Because Schaart et al. (2005) had shown the FaPGIP2 and FaPGIP3 genes to be similar to *F. vesca* and *F. iinumae*, respectively, it was anticipated that the FaPGIP1 sequences might match one of the other diploid species suggested as ancestors of the octoploid species. Potter et al. (2000), using both genomic and chloroplast sequence, identified *F. nubicola* and *F. orientalis* as belonging to the same clade as the cultivated octoploid, whereas DiMeglio and Davis

(unpublished but discussed in Folta and Davis, 2006) suggested that *F. mandshurica* (possibly a diploid form of *F. orientalis*) was a candidate. Yet the sequence from *F. mandshurica* did not match any of the other sequenced alleles, differing from the octoploid sequences at numerous sites, including a 3bp deletion in the coding region. Although the accession intended as *F. nubicola* was later identified as *F. bucharica*, it is in fact the same accession used by both Potter et al. (2000) and DiMeglio and Davis and thus their suggestions should remain valid. However, while resembling both the *F. vesca* alleles and the FaPGIP2 octoploid genes, the *F. bucharica* sequence did not exactly match any of them.

It seems likely that although all the genes examined here are closely related, not all the classes of octoploid alleles represent homoeologous loci, duplicated through polyploidy. Given that PGIP genes have been shown to be tandemly-repeated in nearly every genome in which it has been investigated, including the closely related raspberry (Ramanathan et al., 1997; D'Ovidio et al., 2004; Ferrari et al., 2003), it is not unreasonable to suppose that two or more or more of the *Fragaria* genes described here may represent such a duplication. The FaPGIP2 and FaPGIP3 alleles are more similar than the other two classes, and the close parallels within diploid genomes strongly suggest that these may represent the same locus in different octoploid genomes. Yet FaPGIP1-type genes appear to exist in these diploid species in addition to FaPGIP2 or FaPGIP3-type genes, so this may represent a separate locus. Whether or not the different FaPGIP1 alleles obtained from the octoploid represent variation at a locus within a single genome or in multiple genomes is difficult to ascertain. There is more variation in the FaPGIP1 genes than in the FaPGIP2 or FaPGIP3 genes, but they are more similar to each other than these two classes are to each other. The FaPGIP1b allele appears to occur in all the European cultivars and all the American cultivars surveyed here except 'Treasure', and may

represent a non-polymorphic locus within a specific genome that is not recombining with those that contain the other alleles. The FaPGIP1d form present in 'Treasure' differs from FaPGIPb by only a single nucleotide. It is also possible that some of the of the FaPGIP1-type alleles have been missed by this survey because of variation at one or both of the primer sites.

In the previous study of European octoploids (Schaart et al., 2005), only a single FaPGIP3 allele was identified, and this was found in three of the five European cultivars. A single different FaPGIP3 allele was identified in most of the American cultivars. This suggests a lack of diversity at this locus, and a clear split between the European and American cultivars. Although European octoploids are derived from New World species, European and American breeding programs have engaged in comparatively little exchange of germplasm, as the different environmental conditions require material with different adaptations, and in this case the two groups represent regions of very different climates: European cultivars from Northern and Eastern Europe, and American cultivars from Florida and California.

Methods and Materials

Plant and Genetic Material

A wide range of cultivated and wild germplasm was screened for PGIP alleles. DNA was extracted from young leaves of the *F.* ×*ananassa* cultivars Strawberry Festival, Winter Dawn, and Treasure using the Qiagen DNEasy Plant Mini Kit, according to the manufacturers instructions. Additionally, Dr. Thomas Davis of the University of New Hampshire provided genomic DNA from *F. vesca* 'Pawtuckaway', *F. bucharica* FRA520 (originally considered *F. nubicola*, but reclassified by Staudt (2003)), *F. iinumae* FRA377, and *F. mandshurica* FME, and Dr. Kimberly Lewers of the USDA Agricultural Research Service in Beltsville, Maryland provided genomic DNA of the *F.* ×*ananassa* cultivar Selva. A cDNA library derived from all

plant tissues of salicylate-treated 'Strawberry Festival' (described in Folta et al., 2005) was also used as template.

Cloning and Sequencing of *Fragaria* PGIP Alleles

Oligonucleotide primers were designed based on regions of consensus sequence within four diploid *Fragaria* PGIP sequences available on GenBank: AF196893, AF196892, and AF196891 from several selections of *F. vesca*, and AF196890 from *F. iinumae*. These primers, FrPGIP-F (5'-CTCATGGAAATCCGACGCCG), and FrPGIP-R, (5'-CGACAAGCTTGATCTCACTG) flanked the entire coding region. Because early sequencing and later publications by Mehli et al. (2004) and Schaart et al. (2005) showed that there was little or no variation outside of the intron and LRR region, some PGIP alleles were amplified using primers published by Schaart et al. (2005) that flanked solely these regions: PGIP-seq1 (5'-CCTCCATCGCCAAGCTCAAG), and PGIP-seq2 (5'-CAAGTCCAGTGAGATCAAGC). A FaPGIP1 specific forward primer FaPGIP1-F (5'-CAACGAATTTCCCGTATGCGCG) was also used separately, in conjunction with the PGIP-seq2 reverse. The resulting amplicon from each genotype was cloned into the pCR2100vector, transformed into *E. coli*, and purified using the alkaline lysis plasmid preparation protocol (Bringborm and Doly, 1983). Samples were then quantified via spectrophotometry and submitted for sequencing from the M13 forward and reverse primers. A total of 54 clones were sequenced.

Sequence Analysis

The genomic sequences were aligned using Clustal W software hosted by European Molecular Biology Laboratory – European Bioinformatics Institute (EMBL-EBI) at http://www.ebi.ac.uk/clustalw/, using the default settings. Phylogenetic trees were developed utilizing this software, using the neighbor joining (NJ) method of Saitou and Nei (1987). Nucleotide sequences were translated into amino acids using the Molecular Toolkit software

(http://arbl.cvmbs.colostate.edu/molkit/translate/index.html), hosted by Colorado State University, and splice sites identified according to the mRNA sequence provided in Mehli et al. (2004).

Table 5-1. FaPGIP alleles identified in various octoploid strawberry cultivars.

	FaPGIP isoform											
Cultivar	**1a**	**1b**	**1c**	**1d**	**2a**	**2b**	**2c**	**2d**	**2e**	**3a**	**3b**	**4**
Elsanta[z]	+	+			+							
Korona[z]		+	+			+				+		
Polka[z]		+	+		+	+				+		
Senga Sengana[z]		+	+		+	+	+			+		
Tenira[z]		+			+							
Strawberry Festival	+	+			+						+	
Winter Dawn		+			+			+			+	+
Treasure				+	+							+
Selva		+			+				+		+	

[z] European cultivars from Schaart et al. (2005) presented here for comparison purposes.

Table 5-2. Percentage of identity between PGIP isoforms at the mRNA (standard print) and amino acid (bold face) levels.

	1a	1b	1c	1d	2a	2b	2c	2d	2e	3a	3b	4	Fv	Fi	Fb	Fm	Ri1
FaPGIP1a	—	99	99	97	98	98	98	97	97	96	95	95	98	95	96	96	86
FaPGIP1b	**100**	—	99	99	98	98	98	98	98	96	97	95	98	97	98	97	88
FaPGIP1c	**99**	**99**	—	99	98	98	98	98	98	96	96	95	98	96	98	97	87
FaPGIP1d	**98**	**98**	**97**	—	98	98	99	98	98	96	96	96	98	96	98	97	86
FaPGIP2a	**98**	**98**	**97**	**97**	—	100	99	99	99	97	98	95	100	98	99	98	88
FaPGIP2b	**98**	**98**	**97**	**97**	**100**	—	99	99	99	97	98	95	100	98	99	98	88
FaPGIP2c	**97**	**97**	**97**	**96**	**99**	**99**	—	99	99	97	98	96	99	98	99	98	88
FaPGIP2d	**97**	**97**	**97**	**96**	**97**	**97**	**98**	—	99	97	98	96	99	98	99	99	88
FaPGIP2e	**98**	**98**	**97**	**97**	**100**	**100**	**99**	**97**	—	97	98	96	99	98	99	99	88
FaPGIP3a	**96**	**96**	**95**	**95**	**97**	**97**	**97**	**97**	**97**	—	99	94	97	99	97	96	88
FaPGIP3b	**97**	**97**	**97**	**96**	**98**	**98**	**97**	**97**	**98**	**97**	—	95	98	100	98	97	88
FaPGIP4	**90**	**90**	**90**	**89**	**92**	**92**	**91**	**91**	**92**	**90**	**90**	—	95	95	96	95	85
FvPGIP	**98**	**98**	**97**	**97**	**100**	**100**	**99**	**97**	**100**	**97**	**98**	**92**	—	98	99	98	88
FiPGIP	**97**	**97**	**97**	**96**	**98**	**98**	**97**	**97**	**98**	**97**	**100**	**90**	**98**	—	98	97	88
FbPGIP	**97**	**97**	**96**	**95**	**98**	**98**	**97**	**97**	**98**	**96**	**97**	**90**	**98**	**97**	—	99	87
FmPGIP	**97**	**97**	**97**	**96**	**99**	**99**	**98**	**98**	**99**	**95**	**97**	**92**	**99**	**97**	**97**	—	87
RiPGIP1	**88**	**88**	**88**	**88**	**90**	**90**	**89**	**89**	**90**	**90**	**90**	**82**	**90**	**90**	**88**	**89**	—

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FaPGIP1a      LKMLRLSWNGLSGSVPDFLSQLKNLTFLELNYNNFTGSVPNSLSKLPNLLALHLDRNQLT 60
FaPGIP1c      LKMLRLSWNGLSGSVPDFLSQLKNLTFLELNYNNFTGSVPNSLSKLPNLLALHLDRNQLT 60
FaPGIP2a      LKMLRLSWNGLSGSVPDFLSQLKNLTFLELNYNNFTGSVPSSLSKLPNLLALHLDRNQLT 60
FaPGIP2c      LKMLRLSWNGLSGSVPDFLSQLKNLTFLELNYNNFTGSVPSSLSKLPNLLALHLDRNQLT 60
FaPGIP3       LKMLRLSWNGLSGSVPDFLSQLKNLTFLELNYNNFTGSVPSSLSKLPNLLALHLDRNQLT 60
FaPGIP4       LKMLRFSWNGLCGSFPDFLSQLKNLTFLELNYTNFTGSVPSSLSKVPNWLALHLDRNQLT 60
FbPGIP        LKMLRLSWNGLSGSVPDFLSQLENLTFLELNYNNFTGSVPSSPSKLPNLLALHLDRNQLT 60
FiPGIP        LKMLRLSWNGLSGSVPDFLSQLKNLTFLELNYNNFTGSVPGSLSKLPNLLALHLDRNQLT 60
FmPGIP        LKMLRLSWNGLSGSVPDFLSQLKNLTFLELNYNNFTGSVPSSLSKLPNLLALHLDRNQLT 60
FvPGIP        LKMLRLSWNGLSGSVPDFLSQLKNLTFLELNYNNFTGSVPSSLSKLPNLLALHLDRNQLT 60
              *****:*****.**.*******:*********.*******.* **:** ***********

FaPGIP1a      -GNIPSAYGKFVGTVPDLFLSHNKLTGKIPTSFANMNFDRIDLSRNMLEGDASMIFGMNK 119
FaPGIP1c      -GNIPSAYGKFVGTVPDLFLSHNKLTGKIPTSFANMNFDRLDLSRNMLEGDASMIFGMNK 119
FaPGIP2a      -GNIPSSYGKFVGTVPDLFLSHNKLTGKIPTSFANMNFDRIDLSRNMLEGDASMIFGMNK 119
FaPGIP2c      -GNIPSSYGKFVGTVPDLFLSHNKLTGKIPTSFANMNFDRIDVSRNMLEGDASMIFGMNK 119
FaPGIP3       -GNIPSSFGKFVGTVPDLFLSHNKLTGKIPTSFANMNFDQIDLSSNMLEGDASMIFGMNK 119
FaPGIP4       AGNIPSSYGKLVGTVPDLFLSHNKLTGKIPTSFGNMNFDRIDLSRNMVEGDASMIFGMNK 120
FbPGIP        -GNIPSSYGKFVGTVPDLFLSHNKLTGKIPTSFANMNFDRIDLSRNMLEGDASMIFGMNK 119
FiPGIP        -GNIPSSFGKFVGTVPDLFLSHNKLTGKIPTSFANMNFDRIDLSRNMLEGDASMIFGMNK 119
FmPGIP        -GNIPSSYGKFVGTVPDLFLSHNKLTGKIPTSFANMNFDRIDLSRNMLEGDASMIFGMKK 119
FvPGIP        -GNIPSSYGKFVGTVPDLFLSHNKLTGKIPTSFANMNFDRIDLSRNMLEGDASMIFGMNK 119
              .******::**:**********************.******::*:* **:**********:*

FaPGIP1a      TTQIVDLSRNMLEFDLSKVVFST 142
FaPGIP1c      TTQIVDLSRNMLEFDLSKVVFST 142
FaPGIP2a      TTQIVDLSRNMLEFDLSKVVFST 142
FaPGIP2c      TTQIVDLSRNMLEFDLSKVVFST 142
FaPGIP3       TTQIVDLSRNMLEFDLSKVVFST 142
FaPGIP4       TAQIVDLSRIMLEFDLSKVVFST 143
FbPGIP        TTQIVDLSRNMLEFDLSKVVFST 142
FiPGIP        TTQIVDLSRNMLEFDLSKVVFST 142
FmPGIP        T-QIVDLSRNMLEFDLSKVVFST 141
FvPGIP        TTQIVDLSRNMLEFDLSKVVFST 142
              * ******* *************
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Figure 5-1. Amino acid alignment of the polymorphic portion of a number of *Fragaria* PGIP genes. Shaded areas indicate the xxLxLxx beta-sheet regions. Note that several alleles are omitted because they encode identical proteins to others listed despite differing nucleotide sequences.

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BIOGRAPHICAL SKETCH

Philip J. Stewart was born in Olean, New York, on July 30, 1974. He received his elementary and secondary education at Cuba-Rushford Central School, Cuba, New York, graduating in 1992. Upon graduating, he received a National Merit scholarship to Alfred University, in Alfred, New York, where he obtained a Bachelor of Arts in Biology in 1996. He accepted a job as a research technician with Cornell University, at the New York State Agricultural Experiment Station in Geneva, New York. He spent five years in this position, assisting in the development of wine and table grape cultivars for the Finger Lakes Region and conducting research into bud cold hardiness and grape pedigrees.

In August, 2000, he married Cynthia R. Cowan, in Rochester, New York, and soon returned to school to further study fruit breeding. In 2003 he received a Masters of Science in Horticulture from the University of Arkansas, where he studied disease resistance in blackberries under Dr. John Clark. He began a doctoral program at the University of Florida starting in January of 2004, investigating the molecular biology of strawberries with Kevin Folta and Craig Chandler. During the course of his studies, he and his wife welcomed two daughters Zea, in 2002, and Adina in 2006.

Philip has accepted a job as Strawberry Breeding Manager with Driscoll Strawberry Associates, developing proprietary strawberry varieties for Driscoll's growers in a number of regions of North America. He will begin employment in Watsonville, California upon completion of his degree.