Relating Anthocyanidin Synthase Gene Expression to Floral Color Change in *Saponaria officinalis*

By

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Abstract

Saponaria officinalis is a protandrous common weed that is pollinated primarily by moths. In the male stage, the flower will be white, while in the female stage the flower will be pink. This color change is due to the accumulation of anthocyanins, a common flavonoid, in the petals. These pigments are produced via a biochemical pathway the steps of which are catalyzed by enzymes including anthocyanidin synthase (ANS). The goal of this research was to determine if the change in flower color is accompanied by an increase in expression of the *ANS* gene. One flower from 5 different developmental stages was collected from 10 different plants and their color quantitated by measuring light reflectance. RNA from the petals of each flower was then isolated and *ANS* expression was measured by using qPCR. We found that flower color did significantly increase from male to female phase flowers. Expression of the *ANS* gene was also significantly higher in female phase flowers. Therefore, the transition to pink in female flowers does seem to correspond to an increase in *ANS* expression.

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Acknowledgements

I would like to thank the Strain Honors College for helping with the development of my proposal, providing the funding for this project, and for their support. The biology department of the University of Indianapolis for the use of their facilities and resources. Dr. Davis for being a wonderful mentor. As well as DePauw University collaborators and the previous students who have worked on *S. officinalis*.

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Introduction

Saponaria officinalis, commonly known as bouncing bet, is a common weed found throughout the continental United States, Canada, and Europe. It is a short-lived perennial that is classified within the Caryophyllaceae family (Jabbari et al., 2011). A unique attribute of bouncing bet is its color-changing flowers which are also protandrous. Protandry is a form of dichogamy, in which the stigma does not become receptive before the stamens have released their pollen (Davis et al., 2014). In the bud and male stages of the flower, the petals of each flower are white in color; however, as they transition from male to female, the flower turns pink. Protandry is significant to the fertilization process for *Saponaria officinalis* as it inhibits self-fertilization within flowers by separating the maturation of male and female reproductive units in time. This makes pollination by insects crucial for the successful reproduction of the plant. When insects transport pollen from a male-phase flower on one plant to a female-phase flower on another plant, this results in cross-fertilization. Cross-fertilization is thought to be evolutionarily beneficial as it increases the genetic diversity among the resulting offspring (Lloyd & Schoen, 1992). Furthermore, color change in flowers is found in many plant species and is thought to direct pollinators to reproductively receptive flowers and increase pollination efficiency (Weiss, 1995). Previous research on *S. officinalis* has shown that insects will preferentially visit the white male-phase flowers compared to the pink female-phase flowers (Jabbari et al., 2011). The pollinators may be using white color to distinguish more rewarding flowers as some pollinators collect pollen and there is some evidence that the earlier stage flowers produce slightly more nectar than later stage flowers (Davis et al., 2014). Directing pollinators to male-phase flowers may be beneficial to the plant, as additional visits to flowers

in male stage will increase pollen dispersal and therefore the number of seeds sired; however, increased visits to flowers in the female stage will not necessarily increase the number of seeds set because a flower may only need one or two visits to receive enough pollen that all the eggs within the ovary can be fertilized (Arnold, 1994). Therefore, the coordination of timing between the transition from male to female and color change from white to pink may be an important factor determining reproductive fitness in *S. officinalis*.

The pigments responsible for the pink coloration of the petals in *S. officinalis* are anthocyanins. Anthocyanins are red, blue, and purple plant pigments, classified as flavonoids, and can be found in many common plants (Winkel-Shirley, 2001). The higher the concentration of anthocyanins present, the more color that is expressed in the flower. Anthocyanins are produced via the biochemical pathway shown in Figure 1. Each step within the pathway is controlled by an enzyme; for example, flavonols are expressed when Dihydroflavonol 4-Reductase enzyme (DFR) is produced, while the final step in anthocyanin production is

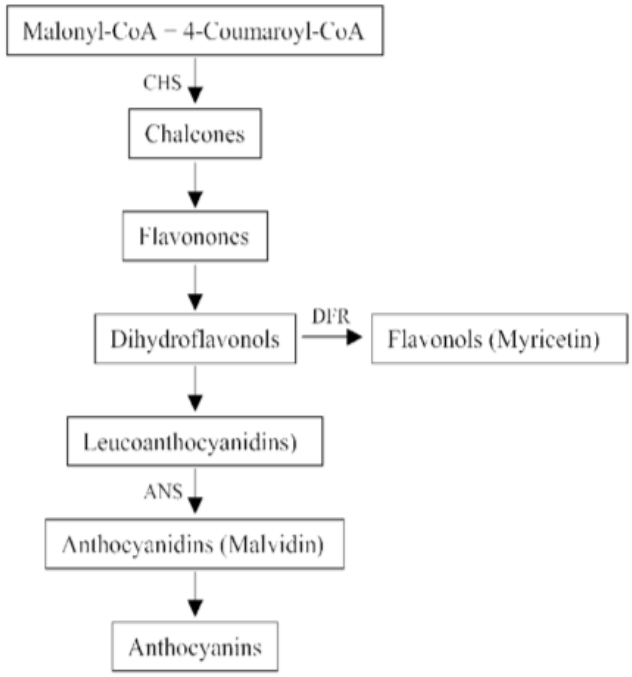

Figure 1: Anthocyanin biosynthetic pathway including enzymes (Farzad et al., 2003)

regulated by the enzyme anthocyanidin synthase (ANS). The production of each of these enzymes is controlled by genes. For example, the ANS enzyme is coded for by the *ANS* gene; therefore, increased or decreased expression of the *ANS* gene can then control how much pigment is produced.

Problem

This project is part of a larger research question that is trying to address if floral color change is potentially an adaptive trait in *S. officinalis*. Pollination by insects has been a major evolutionary pressure in flowering plants as it directly influences the reproductive success of an individual plant. It is theorized that plants have evolved through natural selection to attract pollinators through a variety of methods, including the use of the color of flower petals, the size of the flower, and the timing of which the sexual organs are displayed (Jabbari et al., 2011). On the other side, pollinators have been selected to find rewarding flowers that make themselves more productive while foraging. Since most pollinators make visually oriented selections, selection will favor those individual plants that have floral colors that are most attractive to their pollinators (Weis, 1995). For a trait to evolve through natural selection, the trait must be heritable. Although the production of the enzymes involved in the anthocyanidin synthesis pathway are controlled by genes that are heritable, the timing and level of expression of these genes will also affect whether or not the color change affects pollinator behavior in a manner that increases the fitness of an individual plant. For example, if color change is being regulated by the enzymes later in the pathway, such as *ANS*, depicted in Figure 1, we would expect expression of the earlier genes to remain constant across floral stages. In this manner,

these enzymes would be advancing the production of anthocyanidins to a later stage in the pathway that could be quickly converted to the pink pigmentation with the activation of the later acting *ANS* gene. On the other hand, if the production of the enzymes and pigment precursors are metabolically costly, we might predict that the expression of genes earlier in the pathway would not increase unless one of the environmental factors shown to cause an increase in pigmentation (sun exposure, pollination, etc.) is present.

This project studied the expression of the *ANS* gene throughout the various stages of development, as *Saponaria officinalis* changes from male to female, using real-time quantitative polymerase chain reaction (RT-qPCR). It was hypothesized that if the *ANS* gene is involved in the regulation of floral color change, then there should be an increase in the level of *ANS* expression during flower development.

Background

For this project, I worked under the supervision of Dr. Davis. During semester II of the 2018-2019 academic year, I learned and familiarized myself with the process of real-time quantitative polymerase chain reaction (RT-qPCR). In the traditional process of PCR, a gene to be amplified is chosen, in this case the *ANS* gene, and copies are made in an exponential pattern (Lu et al., 2009). After each cycle, the quantity of the gene is doubled, thus when starting with one copy, after 10 cycles there will be 1024 copies, after 20 cycles there will be $1.048*10^6$ copies, and after 40 cycles, which is the typical number of cycles, there will be $1.099*10^{12}$ copies of the gene. However, around 30 cycles, supplies for the replications will begin to dwindle, resulting in a leveling-off effect and a plateau so that the number of copies will reach a maximum. Traditional PCR only detects the amplified gene at the end of the process. In RT-qPCR, the number of copies of the amplified gene is measured and tracked as the reaction progresses throughout each cycle (Smith et al., 2012). This can be seen using a fluorescent reporter molecule, which will become more intense as the amount of DNA increases. RT-qPCR can be used to measure the amount of starting DNA in a sample, as the greater the number of starting copies of a gene, the fewer cycles of PCR it will take to reach the plateau stage. The central dogma of genetics begins with DNA going through the process of transcription to create RNA, which then goes through translation to become a protein. Genes are expressed by transcription of mRNA from DNA; therefore, the more mRNA copies of a gene present, the more active or "turned on" a gene is. RT-qPCR was used to measure the amount of *ANS* mRNA present in the *S. officinalis* samples.

Method/Procedure

The samples used in this study were taken from *S. officinalis* plants grown in an experimental garden and studied by Dr. Davis in July of 2015 at the DePauw University Nature Park in Greencastle, Indiana. To measure floral color during the gender transition, 6 flowers on 11 different plants of *S. officinalis* were tagged and collected at 6 different developmental stages: bud, early male, late male, early female, mid female, and late female, for a total of 65 flowers. The color of each flower was measured using an Ocean Optics Reflectance Spectrometer™ with a UV-VIS light source (200–800 nm) and fiber optic reflectance probe. Reflectance versus wavelength was plotted to generate a reflectance spectrum (Figure 2), in order to calculate a pinkness index (PI) (Figure 3).

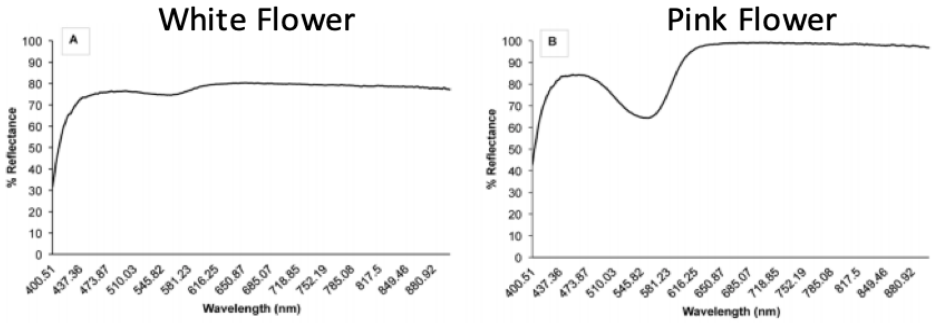

Figure 2: Reflection spectrum of white and pink flowers

$$PI = \frac{\overline{\%R_{540-575nm}}}{\overline{\%R_{455-480and650-700nm}}}$$

Figure 3: Pinkness index equation

To measure ANS expression across stages, 5 flowers on 10 different plants growing in the same experimental garden as above were tagged and collected at five different developmental stages: bud, early male, late male, early female, and late female, for a total of 50 flowers. As the experimental garden was directly adjacent to the Manning field lab of the Nature Park, we were able to flash freeze the petals of each flower in liquid nitrogen and process them directly upon collection. RNA was then isolated from the petals of the flowers

using guanidinium-phenol-chloroform extraction (i.e., TriReagent), labeled and stored in 500mL Trizol within a -80ºC non-frost-free freezer. In order for samples to be put through RT-qPCR, the RNA samples were then converted into complementary DNA (cDNA). This was done through the process of reverse transcription with the enzyme reverse transcriptase (Keyser et al., 2013). Once the cDNA was obtained, the samples were again stored at -80ºC until I used them for RT-qPCR to measure the expression of the *ANS* gene within the sample.

cDNA samples were analyzed with Eco™ Real-Time PCR System by Illumina. This system uses 48-well plates, to allow for 48 reactions to be run simultaneously. For each cDNA sample, 3 replicate PCR reactions were run using primers specific to the *ANS* gene and 3 replicate PCR reactions were run using primers specific to the Actin gene (*ACT*). Actin is a protein that makes up the cytoskeleton of cells and is therefore expected to be expressed by all cells at roughly the same level, so it is used to obtain a base-line value to which to compare the *ANS* levels. A typical 48-well plate was used and set-up as shown in Figure 4. Each of the 48 wells was loaded with 6 µL of a master mix containing iQ SYBR® Green Supermix, which contains the reporter molecule and PCR reagents and either 10µM ANS primers or 10µM ACT primers. Column 1 of each plate was the no template control, containing only primer mixture and 6 µL of nuclease-free water. Columns 2 and 3 were a

		1	2	3	4	5	6	7	8
ANS PRIMERS	A	NTC	0.1	0.0001	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
	B	NTC	0.01	0.00001	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
	C	NTC	0.001	0.000001	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
ACT PRIMERS	D	NTC	0.1	0.0001	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
	E	NTC	0.01	0.00001	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
	F	NTC	0.001	0.000001	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5

Figure 4: 48-well RT-qPCR plate set-up

serial dilution, starting with 2 μL of original sample cDNA added to 18 μL of nuclease-free water (a 1:10 dilution), and serially diluted to the amounts shown in the figure. Columns 4 through 8 were loaded with 6 μL of 1:10 diluted cDNA samples. The plate was then sealed and placed in the Illumina machine and ran for 40 cycles of PCR that consisted of 60 seconds at 95°C, 60 seconds at 54°C, and 120 seconds at 72°C. The difference between average C_T value of the 3 replicates for each sample using *ANS* and using *ACT* primers was then determined (the delta value). The largest of these delta values (3.22) was then subtracted from each of these delta values, creating the Delta Delta value. The delta delta value was then converted into a log value to calculate the ratio of ANS:ACT expression by the formula $2^{-\Delta\Delta}$. The results were then examined to determine if there was an association between the *ANS* gene expression and the color change of the flower.

Results

Pinkness Index of Floral Stages

All data were analyzed with IBM SPSS version 25 software. For flower color, a one-way ANOVA (Table 1) found a significant difference in pinkness index across floral stages. A pairwise Tukey post-HOC test was run to determine which of the stages were different from each other. This corresponds to the graph in Figure 5 and the letters differentiate between the stages that are significantly different from each other. The bud stage (A) is significantly different from all the female stages; however, early and late staminate stages (AB) are not significantly different from the bud stage or the early pistillate (B) but is significantly different from mid and late pistillate stages (C). The early pistillate stage (B) is

Table 1: One way ANOVA to determine if there is a significant difference in pinkness index

ANOVA

PinkIndex

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.455	5	.091	10.990	.000
Within Groups	.497	60	.008		
Total	.951	65			

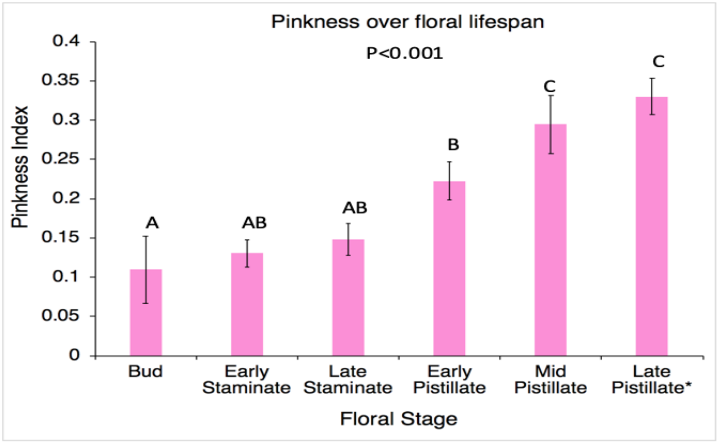

Figure 5: Pinkness over floral lifespan corresponding to statistical analysis in Table 2.

Table 2: Post-HOC tests indicating which floral stages are different from each other

Homogeneous Subsets

PinkIndex

Tukey HSD[a,b]

Stageno	N	Subset for alpha = 0.05		
		1	2	3
1.00	8	.105834244		
2.00	12	.130292049	.130292049	
3.00	12	.147812866	.147812866	
4.00	12		.222155645	.222155645
5.00	10			.294243971
6.00	12			.329998536
Sig.		.891	.194	.081

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 10.746.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

significantly different from bud (A) and mid and late pistillate stages (C). The mid and late pistillate stages (C) are significantly different from all other stages.

ANS Expression in Different Floral Stages

Three outliers were found, as shown in Figure 6, and removed from the data before analysis. To determine if there was a significant difference in *ANS* gene expression between floral stages and plant number, a two-way ANOVA was run with floral stage considered a fixed dependent variable and plant number as a random dependent variable. Table 3 shows the results without the bud stage included in the calculations; however, Table 4 does include the bud stage. The bud stage is not male or female so the results were separated. As seen in both tables, there is no significant difference when looking at both flower number and flower stage together. Since there was no significant interaction between floral

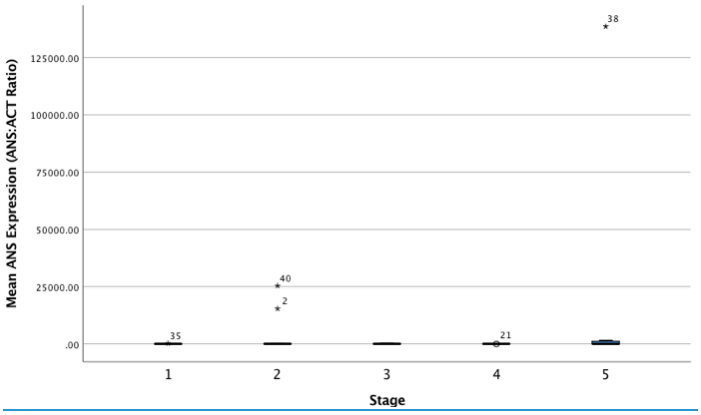

Figure 6: Outlier test

Table 3: Two-way ANOVA to determine if ANS expression is significantly different between floral stage and plant number (bud stage not included)

Tests of Between-Subjects Effects

Dependent Variable: Ratio

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	304091.280	1	304091.280	3.729	.085
	Error	735803.438	9.023	81550.361[a]		
Stage	Hypothesis	570184.596	3	190061.532	1.923	.167
	Error	1581627.05	16	98851.690[b]		
FlowerNumber110	Hypothesis	733791.421	9	81532.380	.825	.603
	Error	1581627.05	16	98851.690[b]		
Stage * FlowerNumber110	Hypothesis	1581627.05	16	98851.690	.	.
	Error	.000	0	.[c]		

a. .999 MS(FlowerNumber110) + .001 MS(Stage * FlowerNumber110)
b. MS(Stage * FlowerNumber110)
c. MS(Error)

Table 4: Two-way ANOVA to determine if ANS expression is significantly different between floral stage and plant number (bud stage included)

Tests of Between-Subjects Effects

Dependent Variable: Ratio

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	378600.179	1	378600.179	4.545	.061
	Error	762564.532	9.154	83306.213[a]		
Stage	Hypothesis	576498.726	4	144124.682	1.866	.154
	Error	1621569.12	21	77217.577[b]		
FlowerNumber110	Hypothesis	750260.031	9	83362.226	1.080	.417
	Error	1621569.12	21	77217.577[b]		
Stage * FlowerNumber110	Hypothesis	1621569.12	21	77217.577	.	.
	Error	.000	0	.[c]		

a. .991 MS(FlowerNumber110) + .009 MS(Stage * FlowerNumber110)
b. MS(Stage * FlowerNumber110)
c. MS(Error)

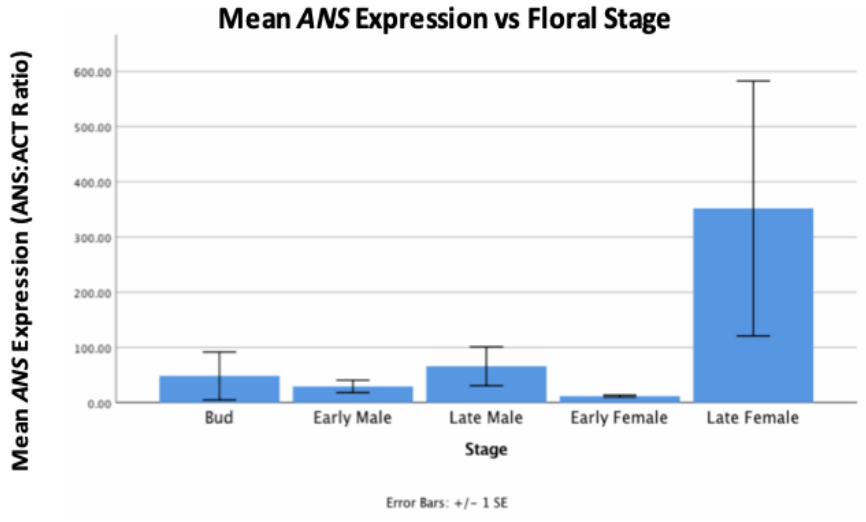

Figure 7: Pooled data for plants of each stage and only looking effect of stage

stage and plant number, the data were pooled across plants for each stage and just the effect of stage was analyzed; however, there was still no significant difference in *ANS* expression between floral stages (Figure 7) (P value 0.162), although the trend for higher expression was what we predicted. This may be due to low sample size, since we only analyzed 10 flowers per stage. Therefore, the data were pooled between male and female stages. When comparing just male vs. female stage, Figure 8 shows that male stage flowers had significantly less *ANS* expression than female stage flowers (P value 0.014).

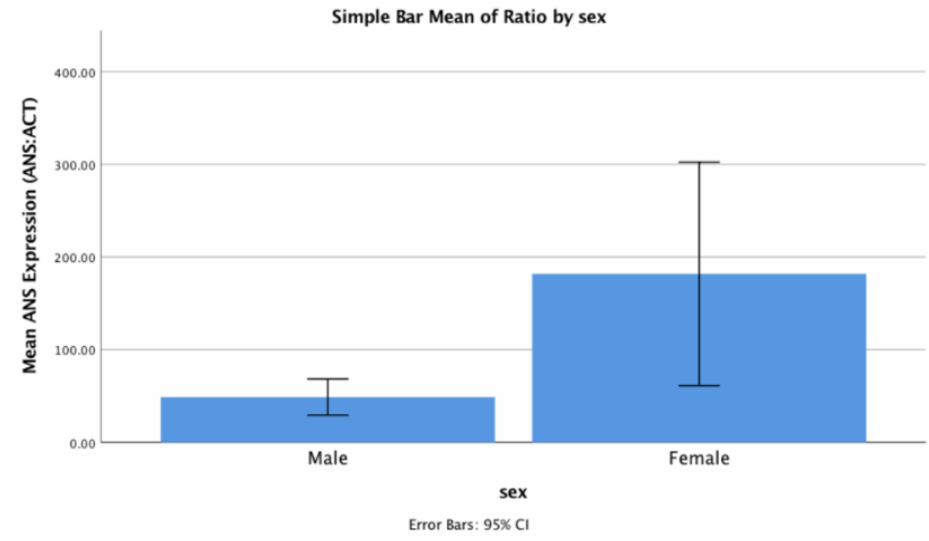

Figure 8: Pooled data for male and female stage plants

Analysis/Conclusion

The hypothesis, if color change is being regulated by *ANS* gene in the anthocyanin biosynthetic pathway, then there should be an increase in the level of *ANS* expression as *S. officinalis* proceeds through its floral stages and transitions from white to pink, was supported. *S. officinalis* flowers do significantly increase in pinkness from early male to late female stages. Despite no significant difference being found in *ANS* expression between the individual floral stages, there was a significant difference in *ANS* expression between male-phase and female-phase flowers. Since only 10 flowers per floral stage were sampled in this study, increasing the sample size could allow us to detect differences between the individual stages to help narrow down the timing of when the increase in *ANS* gene expression occurs.

Furthermore, these data suggest that increased expression of the *ANS* gene is directly associated with the increasing amount of anthocyanins in *S. officinalis* flowers. Regulating color expression via the *ANS* gene, which has a late role in the anthocyanin biosynthetic pathway, may have significant implications. Similar to this study, Farzad et al. (2003) found that, during flower development in color changing violets, *ANS* expression increased as the flower changed from white to a purple color; but, *CHS* and *DFR,* which act earlier in the pathway (Figure 1), expression stayed the same. However, there was an increase in expression in *CHS, DFR,* and *ANS* in response to increased light and pollination. This suggests *ANS* is regulating color change during ontogeny, but all three are involved in generating a response to environment. Pollination and exposure to light have also been shown to trigger floral color change in other species (Woltering et al., 1995). Both of these factors could also be contributing to floral color change in *S. officinalis* and the expression of DFR across floral

stages are currently being examined. The results of this experiment could contribute to establishing a correlation between flower gender, color, and pollinator attraction to a specific floral stage. If, as in violets (Farzad et al., 2003), genes that code for enzymes that act early in the production of anthocyanins are already being expressed in the bud and male stages, then the color transition from white to pink could occur rapidly with the activation of the *ANS* gene, which acts late in the pathway. Floral color change has evolved multiple times in angiosperms across many different taxa. It is thought to be an adaptation that influences pollinator behavior. The retaining of color changed flowers increases the overall display size of the inflorescence, thereby attracting a greater number of insect pollinators seeing the display from a distance. Once the pollinators have reached the inflorescence, the color difference may direct the pollinators to more rewarding flowers (Weiss, 1995). This could reduce the probability of self-pollination and increase pollen dispersal by increasing the number of initial visits to an inflorescence, but decreasing the number of flowers within an inflorescence an insect visits, transferring pollen from one flower to another of the same individual (geitonogamous pollination). In *S. officinalis*, floral color change has been shown to increase the probability of pollinator visits to male-phase white flowers over female-phase pink flowers (Davis et al., 2014). Therefore, floral color change in *S. officinalis* may be an adaptive trait that increases cross-pollination via pollinator signaling.

Reflection

The goals of this project were met as I learned how to perform RT-qPCR, presented my findings to the Biology Department in an oral presentation in the format of a professional scientific talk, and was able to achieve statistically significant results. There were a few difficulties in this project. The first being time, as I am graduating in December so the actual research was done within one semester. A second complication was the amount of DNA I had available. The original samples I was supposed to work with were destroyed when the Biology Department's -80^{o}C freezer malfunctioned. Luckily, there were replicates of the samples at DePauw University and I was able to use those; however, there were a few samples missing and the amount of cDNA left in some was just enough to do two RT-qPCR runs. This project was personally important to me because this is a major life milestone that I completed, one of which I have had planned for years, and it puts me one step closer to graduation. It was also professionally important to me as this is what I want to do in the future. I plan on attending graduate school for molecular biology, a program which is heavy on gene regulation, biosynthetic pathways, and lab research. This project was a great preparatory experience. I improved my analytical thinking skills through examining my data and interpreting how the data solves the addressed problem. I also boosted my skills in problem solving as there were a few trials with messy data and the issue had to be identified before those samples were run again to achieve accurate data. Furthermore, I had to be open-minded to taking critique throughout the entire process of this project since it is given to help evolve my skills needed for my future.

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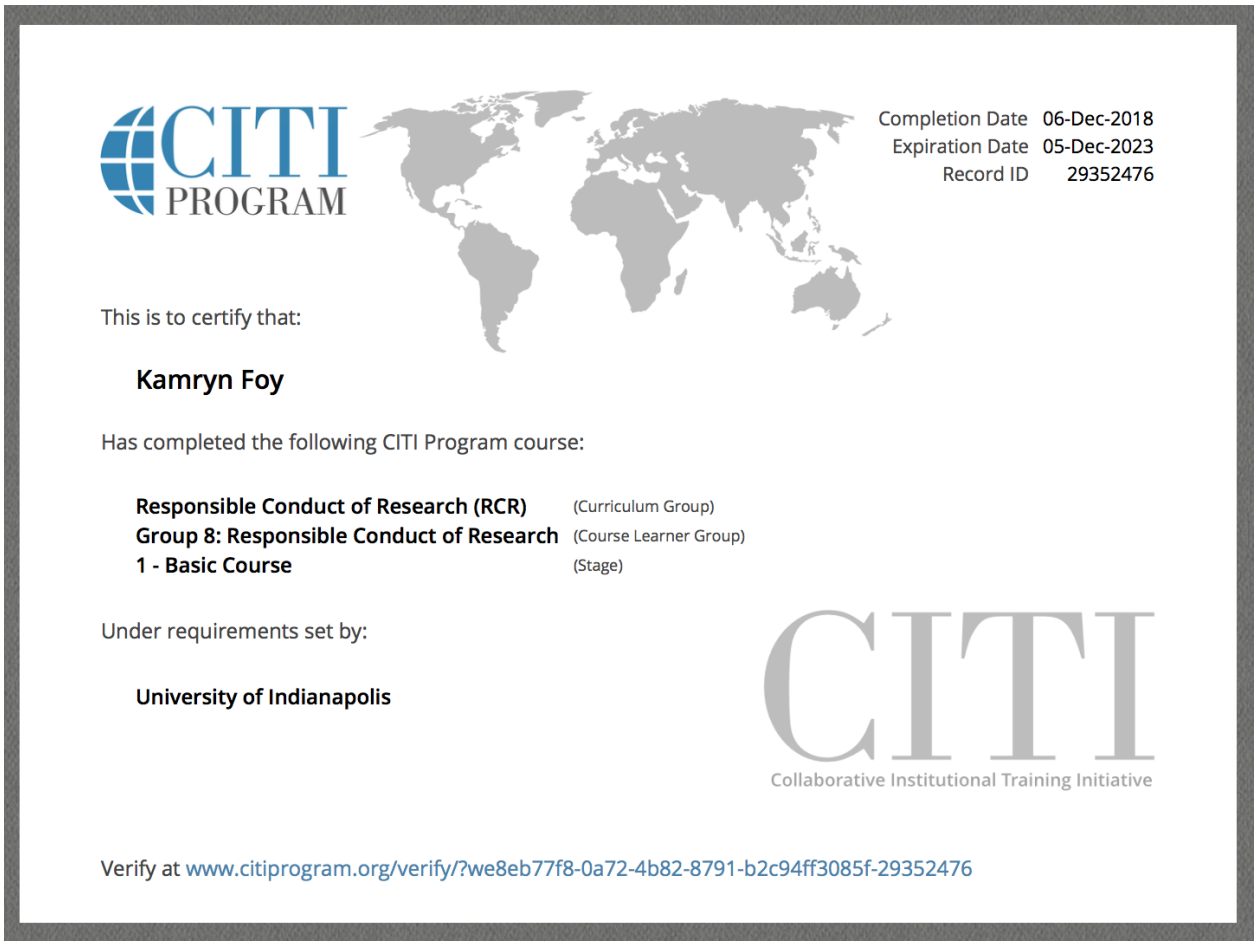
Appendix



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