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A Preliminary Study on the Role of Enhancer of Flavonoid Production (EFP) in Flavonoid Biosynthesis

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A Preliminary Study on the Role of *Enhancer of Flavonoid Production* (*EFP*) in Flavonoid Biosynthesis

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ABSTRACT

Flavonoids are involved in a variety of biological roles ranging from pathogen protection, pigment intensity, antioxidant effects, and even prevention from cancer or cardiovascular diseases. Because of the diverse and beneficial functions that flavonoids have, the flavonoid biosynthetic pathway has been well studied. Recently, a gene called the *Enhancer of Flavonoid Production* (*EFP*) was discovered when mutations in this gene caused the Japanese morning glory flowers to exhibit a pale-colored phenotype. Although *EFP* is known to increase flavonoid production, the direct mechanism to how *EFP* enhances enzymes in the flavonoid biosynthetic pathway has yet to be discovered. It has been found that the *EFP* gene encodes for a chalcone isomerase (CHI) related protein. Chalcone isomerase is the enzyme involved in the second committed step of the flavonoid biosynthetic pathway. The enzyme preceding chalcone isomerase is known as chalcone synthase (CHS) which functions to produce chalcones, an aromatic ketone that is a precursor to flavonoids. Because *EFP* encodes for a CHI related gene, EFP is likely to enhance enzymes involved in the earlier steps of the pathway such as CHI and CHS. To observe if an interaction exists between EFP and CHI or CHS, plasmids were constructed for each gene. The plasmid for *EFP* was then agroinfiltrated into *Mimulus lewisii* LF10 mutants that had a mutation in the *EFP* gene to potentially rescue the wild type phenotype. If flavonoid production can be regulated by EFP, then this could lead to many future possibilities, especially involving health and medical benefits.

INTRODUCTION

Flavonoids are metabolites that are commonly found in the leaves, flowers, and seeds of plants. Flavonoids have a basic structure which typically includes a 15 carbon skeleton that consists of two phenyl rings and a heterocyclic ring. Isomers of this structure result in subgroups of flavonoids such as anthocyanins, flavanols, and proanthocyanins (Winkley-Shirley 2001; Jiang et al. 2015). Flavonoid chemicals are involved in various roles, one of which being the protection of plants. These metabolites provide resistance to pathogens and defend plants from UV radiation. Additionally, flavonoids have also been known to be beneficial to human health as they reduce the production of reactive oxidative species (ROS), serving as an antioxidant (Jiang et al. 2015; Mierziak et al. 2014). The importance of flavonoids in plant physiology and human health has allowed the flavonoid biosynthetic pathway to be extensively studied.

As mentioned earlier, one of the products of the biosynthetic pathway is anthocyanin, molecules responsible for pigmentation. In the flavonoid biosynthetic pathway, chalcone synthase catalyzes the first committed step to form chalcone. Chalcone isomerase then isomerizes the chalcone into an early intermediate of flavanone. Afterwards, an –OH group is introduced to the flavanone by flavanone 3-beta hydroxylase. Then two enzymes, dihydroflavanol 4-reductase and anthocyanidin synthase, react with dihydroflavnols to make anthocyanins. Finally, the anthocyanins are glycosylated by flavonoid 3-O-glucotransferase to generate stable molecules of anthocyanins (Ferreyra et al. 2012; Jiang et al. 2015).

Recently, research has found three mutations in a gene that generated a reduction in flower pigmentation in Japanese morning glory, resulting in a pale-colored pigmentation. This gene was discovered to be the *enhancer of flavonoid production (EFP)* gene. Compared to plants in which *EFP* was knockdown, *EFP* significantly increased the production of anthocyanin. Subsequently, *EFP* has been found to encode for a *chalcone isomerase* type protein, which is one of the enzymes involved in the biosynthetic pathway (Morita et al. 2014). With the discovery of this new gene, multiple studies were done to determine the mechanism in how EFP enhances anthocyanin production.

The *EFP* gene was first discovered as a spontaneous mutation that would induce a pale color phenotype in Japanese morning glory *(Ipomoea nil)*. To check which gene was responsible, a simplified transposon display was performed to isolate the mutant gene tagged by transposable elements in the *Tpn1* family (Fukada-Tanaka et al. 2001; Inagaki et al. 1994). The mutant gene was found to be the *efp-1* allele. To confirm that *efp-1* mutations are responsible for the pale colored phenotypes, two germinal revertants of *efp-1* showed the normal pink phenotype. The only difference is that the germinal revertants had lost a *Tpn13* insertion within the gene (Morita et al. 2014). This shows that the *Tpn13* insertion into the *EFP* promoter induced the *efp-1* mutation.

Now that the *EFP* gene has been shown to affect flower pigmentation, the next step would be to see how this gene is involved in the flavonoid biosynthetic pathway. Previous research has shown that the *efp-1* mutants do not play a significant role in

anthocyanin production. This was because there were no changes in the structural gene expressions when comparing the wild-type and *efp-1*. This indicates that it is highly unlikely that the EFP protein could act as a transcriptional regulator of anthocyanin production. Furthermore, *EFP* encodes for a CHI-related protein so it is unlikely for the gene to be involved in affecting anthocyanin transport through vacuoles (Morita et al. 2014). If *EFP* is not essential for anthocyanin production, then the conclusion would be that this gene plays a role earlier in the biosynthetic pathway for flavonoid synthesis.

Additionally, apart from the Ipomoea *EFP* mutants showing a decrease in the levels of flavonoids, there was also a reduction in flavones and flavonols. Since flavones and flavonols are generated after the second committed step in the biosynthetic pathway, it is likely that EFP enhances the initial enzymes involved in the pathway. Therefore, EFP is more likely to be involved in CHS or CHI activity rather than enzymes downstream of the pathway such as F3H. Additionally, although *EFP* encodes for a CHI related gene, it is unlikely that EFP alters the activity of CHI. This is because no chalcone 2-O glucoside was found in the *efp-1* mutants, and this glucoside is typically found in Ipomoea *CHI* mutants (Morita et al. 2014). Therefore, it is reasonable to hypothesize that EFP acts as an enhancer for CHS to promote flavonoid production and flower intensity. In the early steps of the biosynthetic pathway, the enzymes involved form enzyme complexes to make sure sufficient flavonoid production. It would make sense that EFP could be involved in interacting with these enzyme complexes to channel intermediates to influence flavonoid production.

Since it is likely that EFP enhances the initial enzymes in the flavonoid biosynthetic pathway, an experiment is being performed to see how a stable transformation of EFP with either CHS or CHI will affect the pigment intensity and flavonoid production of wild type *Mimulus lewisii* LF10 flowers (Yuan et al. 2013a). The *Mimulus* species was chosen because it serves as an excellent model to study developmental genetics, including pigmentation and anthocyanin patterning (Yuan et al. 2013a). In this case, if EFP enhances either CHS or CHI, it should increase anthocyanin production and generate an overexpression phenotype.

Additionally, our lab performed a large-scale ethyl methanesulfonate (EMS) mutagenesis experiment using *M. lewisii* inbred line LF10. The purpose of this was to generate new phenotypes that could be further studied (Owen and Bradshaw 2011). A few of the mutants showed a white colored phenotype as opposed to the light pink phenotype in wild type LF10. Further research was done to show that there was a mutation in the *EFP* gene. To confirm that *EFP* is the causal gene for this white phenotype, we did a stable transformation of EFP into these mutant plants to attempt to rescue the LF10 wild type phenotype.

By learning more about the *EFP* gene, we could potentially regulate and optimize the production of flavonoids in plants. Additionally, if EFP does enhance the activity of *CHS*, then this could be crucial in providing protection to plants from biotic and abiotic stresses. This is because CHS expression has been known to protect plants from UV light

and microbial pathogens. Therefore, there are many future applications that can be explored by learning the mechanism in how EFP enhances flavonoid production.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The *Mimulus lewisii* plants were grown at the University of Connecticut research greenhouse. The plants were grown under natural sunlight and sodium vapor lights. The LF10 inbred line was described in (Yuan et al. 2013a). A large-scale ethyl methanesulfonate (EMS) mutagenesis experiment using M. lewisii inbred line LF10 was performed. A few of the mutants showed a pale white flower color. A homolog of the *EFP* gene was sequenced in these mutants to show a mutation in the *EFP* gene.

Plasmid Construction

In order to know if EFP enhances either CHI or CHS, the first step was to isolate *EFP, CHI* and *CHS* sequences from the *Mimulus lewisii* LF10 species. A Phusion PCR was done to isolate the desired sequences with primers EFP_cdsF (5'-caccATGGGTACTGAAGCCGAGTA-3')/ EFP_cdsR (5'-TTTAGATAATTGAAGAGAAAGAGC-3'), CHI_cdsF (5'-caccATGTCGGCACCGCCGTCGGTGA-3')/CHI_cdsR (5'-GTTTTCCTGAGGTTTTGCAGCAG-3'), and CHS_cdsF (5'-caccATGGCCAGCATTGAGGAGATTC-3')/CHS_cdsR (5'-GTTGATRGCGACACTATGCAGC-3'). Afterwards, the Phusion PCR products were purified to remove any primers and other impurities from the PCR. The purified DNA products were run on a gel to check if there are single bands. Then, the PCR products for *EFP, CHI* and *CHS* were inserted into a

TOPO vector (Invitrogen) via a pENTR/D-TOPO cloning reaction. After the mixtures have spent about 30 minutes incubating at room temperature, they were transformed into commercial One Shot Top10 *E. coli* competent cells. After the transformation, around 200 µl of the products were spread on kanamycin antibiotic plates and incubated overnight at 37˚C.

After colonies have grown out, a colony PCR was done on the colonies with the M13F primer and the respective cdsR primers for *EFP, CHS,* and *CHI* to check the orientation of insertion. A replica plate was made of the selective colonies so a M13F/R colony PCR could be subsequently performed to check for colonies that include the designated insertion for *CHI, CHS,* or *EFP*. After screening for positive colonies, two colonies with the insert from each gene were selected to isolate the plasmid. The plasmid isolation started by placing 3 mL of Luria-Bertani (LB) broth and 3µl of kanamycin in a 15 mL Falcon tube. The colony was added and the tubes were placed on the shaker to incubate overnight at 37˚C. Then, the plasmid was isolated via a Plasmid Mini-prep kit.

Once the final plasmid of *CHI, CHS,* and *EFP* were obtained, a Phusion PCR was performed on each of the DNA products with the M13F/R primers to check for the insert and prepare for a LR clonase reaction. After the Phusion PCR, the product was gel purified to clean up any impurities. Then a LR reaction was performed where *CHS* was recombined in the pEarleyGate 202YC vector, CHI was recombined in the pEarleyGate 201YN and pEarleyGate 202YC vector, and EFP was recombined in the pEarleyGate 100,

pEarleyGate 101 and pEarleyGate 201YN vector. The pEarleyGate vectors use a virus promoter, CaMV 35S, to drive transgene expression (Earley et al. 2006). The vector p101 includes the EYFP fluorescence protein whereas p201YN and p202YC each have half of the sequence for EYFP protein. The EYFP fluorescence protein allows us to view the localization of the proteins under the confocal microscope. Following the LR recombination reaction, the products were transformed into *E. coli* competent cells and subsequently plated onto kanamycin plates to incubate at 37˚C overnight. Afterwards, a colony PCR was performed to check for the insertion and if the insertion is at the correct orientation. For the colony PCR, *CHI* had the opposite orientation and *CHS* had no colony with the insertion. The *EFP* colonies were correct so two colonies from *EFP* p201YN, *EFP* p101 and *EFP* p100 were selected and isolated. The isolation of the final plasmid followed the same procedure mentioned beforehand where they were placed in LB broth and kanamycin and incubated overnight. Then, a Miniprep was performed to isolate the final plasmids for *EFP* p201YN, *EFP* p101 and *EFP* p100. The final plasmid was sequenced by the Sanger method to verify the orientation and to check that no mutations are present.

Agroinfiltration of M. lewisii leaves (Transient Assay)

Once the *EFP* plasmids have been confirmed to have no mutations, *EFP* p101 and *NEGAN* p100 were transformed into *Agrobacterium tumefaciens* strain GV3101. *NEGAN* is nectar guide anthocyanin, an anthocyanin activating *R2R3-MYB* gene (Yuan et al. 2014). *NEGAN* was used because it contains a visual marker (*NEGAN-mGFP5*) to show

areas of the leaves where infiltration has occurred. The final plasmid was transformed into agro competent cells and plated on plates that included kanamycin, gentamycin, and rifampicin antibiotics to incubate for 2 days at 28˚C. After, an agro preparation was performed for *EFP* p100 and *NEGAN* p100. For the agro preparation, two agro colonies were selected from each of the plates and inoculated into separate 5mL tubes containing LB, kanamycin (50mg/L), gentamycin (50mg/L), and rifampicin (25mg/L). Then, the tubes are put on the shaker for 12-16 hours and then centrifuged for 20 minutes at 6000 rpm. After the centrifugation, the liquid of each of the tubes is poured off and the agrobacterium cells of the *EFP* p101 and *NEGAN* p100 are combined by the addition of the resuspension solution. The resuspension solution included 25 ml 5 % sucrose solution along with 0.1 M acetosyringone and 0.1 % Silwet L-77 (Ding & Yuan 2016). Finally, infiltration was performed by pushing the mixture of *EFP* p101 and *NEGAN* p100 agrobacterium solution gently into the lower side of the leaf lamina into wild type *Mimulus* LF10 by using a needleless syringe (Becton, Dickinson and Company). Typically, two spots on the leaves were infiltrated to assure that enough of the agrobacterium has entered the leaves.

In planta transformation

Plant transformation was performed by using the vacuum filtration (Bechtold et al. 1993) method and floral spray (Chung et al. 2000). The same method explained beforehand in the transient assay was used to prepare the agrobacteria for infiltration. However, this time *EFP* p100 was transformed into the *tumefaciens* strain instead of *EFP*

p101 because EYFP fluorescence is no longer needed. The infiltration was performed on the mutant flowers that resulted in an *EFP* mutation from the EMS screening. The transformation was done when the mutants had approximately 10-15 small flower buds. The buds were initially sprayed with the inoculation solution. Then, the plants were placed into a vacuum chamber where 28-30 Hg of pressure was applied for roughly 3 minutes. The infiltrated plants were then placed under plastic domes for 1 day to maintain high levels of humidity (Yuan et al. 2013b). Once the flowers started opening, self-pollination was performed for approximately two weeks. The T_1 seeds were then collected and planted on flats for transgenic screening by herbicide spray.

RESULTS

Mutant Screening

When the *EFP* gene for the selected mutants with the white colored phenotype was sequenced, a missense mutation was detected.

Plasmid Construction and Sequencing

Previous studies have shown that it is likely for EFP to enhance an earlier enzyme involved in the flavonoid biosynthetic pathway. Therefore, the first two enzymes responsible for the committed steps in the pathway (CHS and CHI) were isolated to potentially see if they interact with EFP. The genes *CHI*, *CHS* and *EFP* were isolated from a complementary DNA sample of *Mimulus LF10* corolla by a Phusion PCR. After PCR purification, the samples were run on a gel and they were found to be all single bands.

EFP was around 700 bp, *CHI* was around 800 bp, and *CHS* was found to be around 1200 bp.

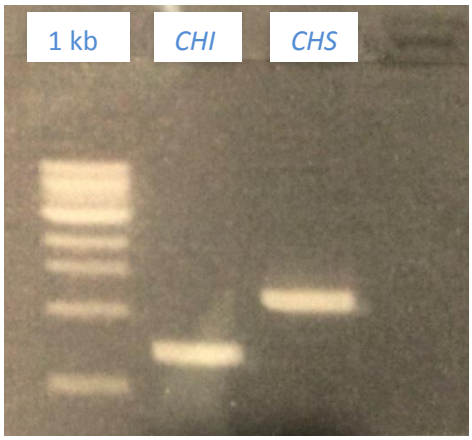

Fig 1: Shows the DNA samples of *CHI* and *CHS* from the Phusion PCR after PCR purification. Single bands are present. The 1 kb ladder shows *CHI* to be around 800 bp and *CHS* to be around 1100 bp.

The sequences of the entry clones for *EFP, CHS, and CHI* all had the correct orientation by having the correct forward and reverse reads for the M13F (5' – TGTAAAACGACGGCCAGTCT -3') and M13R (5'-CAGGAAACAGCTATGACCAT-3'). Additionally, the final plasmid from the LR reaction was also sequenced to check for mutations and orientation. The *CHS* gene was unable to be sequenced due to a lack of insert following the LR reaction. However, p201YN *CHI*, p202YC *CHI*, and p101 *EFP* were all sequenced and had no mutations and the correct orientation.

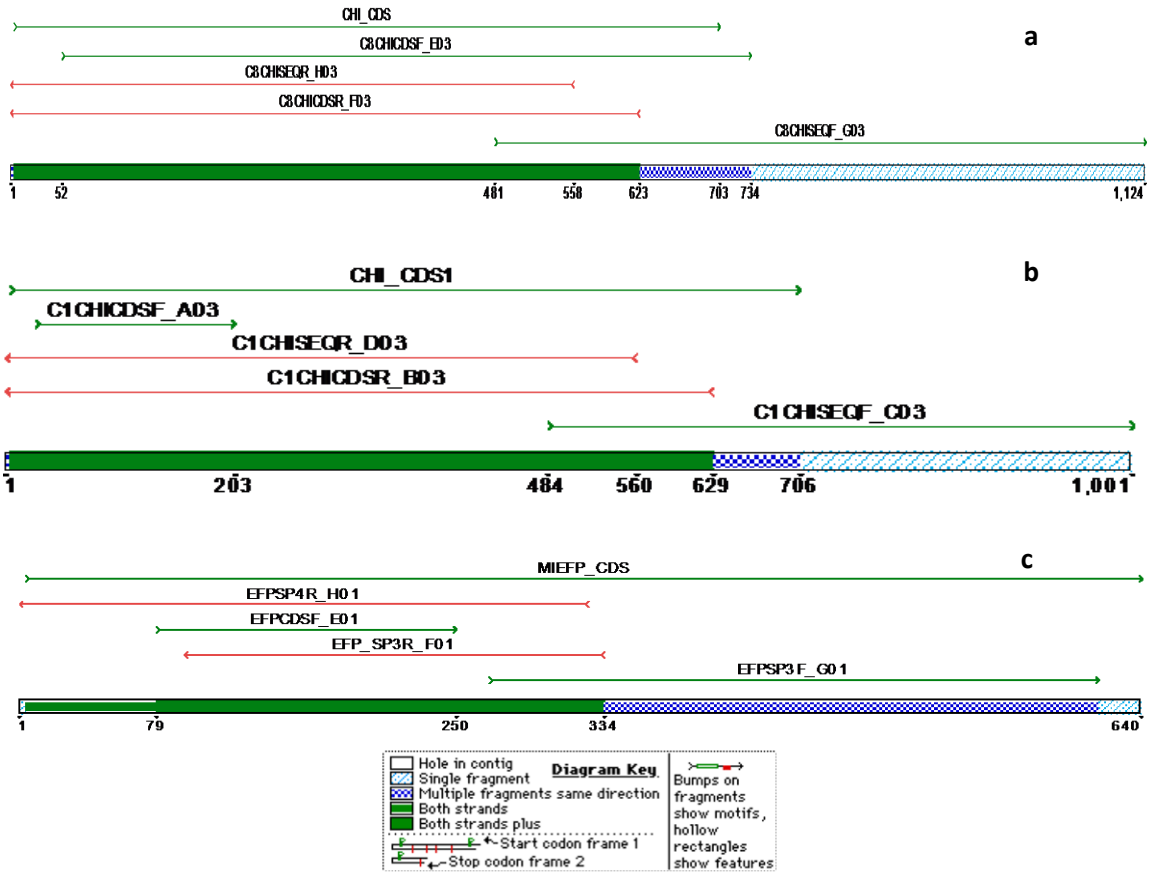

Fig 2: Shows the primers used in the sequencing of *CHI* p201YN, *CHI* p202YC, and *EFP* p101. Used Sequencher DNA sequence Analysis software to view which sections of the gene each primer sequenced. **a** Sequenced 1124 bp of the final plasmid for CHI p201YN. **b** Sequenced 1,001 bp of the final plasmid for CHI p202YC. **c** Sequenced 640 bp of the final plasmid for EFP p101.

Transient Assay

Once the sequences had been verified, a transient assay was performed where *EFP* p101 and *NEGAN* p100 were transformed in *Agrobacterium tumefaciens* for agroinfiltration. Then *EFP* p101 and *NEGAN* p100 were infiltrated into the leaf lamina of *Mimulus lewisii* LF10. After 3 to 4 days, the leaves showed anthocyanin spots due to the visual marker in *35S: NEGAN-mGFP5* construct (Ding and Yuan 2016). The leaves were

viewed under a confocal microscope and the fluorescence images were acquired using a Nikon A1R confocal laser scanning microscope equipped with a 60X water immersion objective (Ding and Yuan 2016). The results of the transient assay show both nuclear and cell membrane localization for EFP.

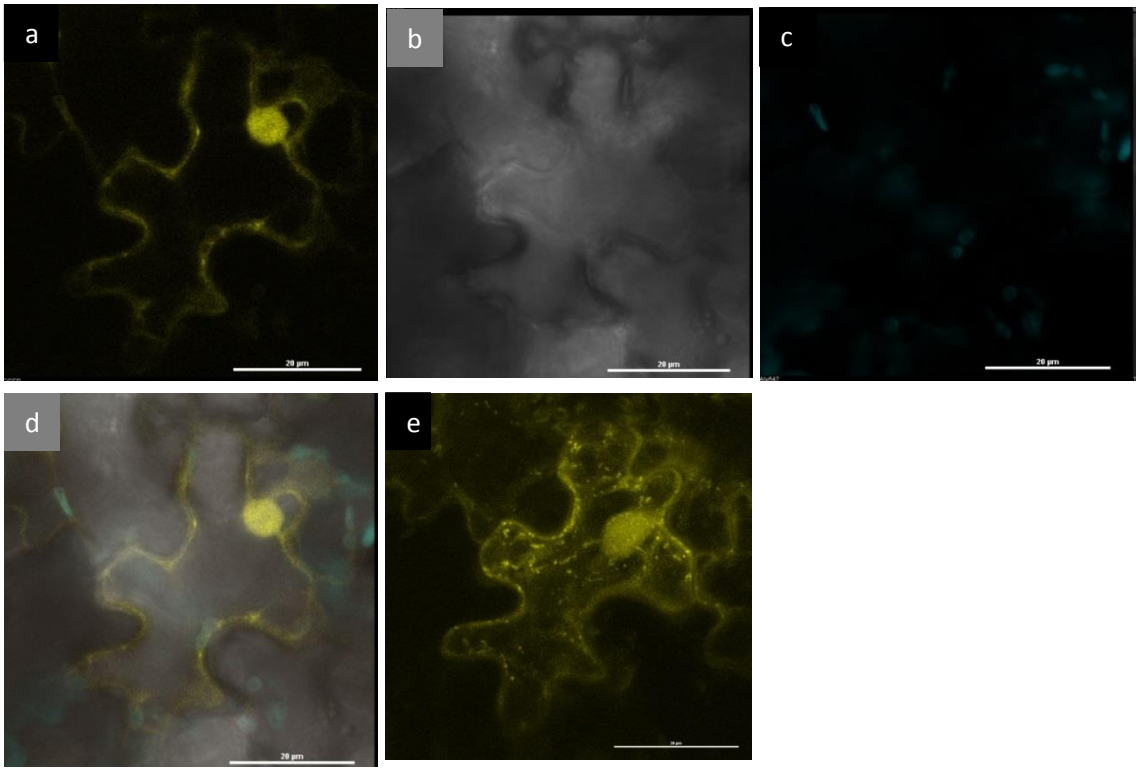

Fig 3: Expression of *35S:EFP-EGFP* in an agroinfilatrated *M. lewisii* leaf. Fluorescence shows successful infiltration of p101 *EFP* and p100 *NEGAN*. **a** An epidermal cell shown under the green channel. **b** The same cell under transmitted light. **c** The same cell under the Alx647 channel for detecting chlorophyll autofluorescence. **d** The merged image of panel a–c. **e** Superposition of a series of confocal optical sections (Ding and Yuan 2016).

Stable Transformation

After the vacuum infiltration, we are currently waiting for the T_1 flowers to grow to see how *EFP* impacts color intensity and anthocyanin production for the EMS induced mutant. By infiltrating EFP into the *efp* mutant, we wanted to test whether EFP

functions to enhance flavonoid production and whether it can rescue the phenotype in the mutant background.

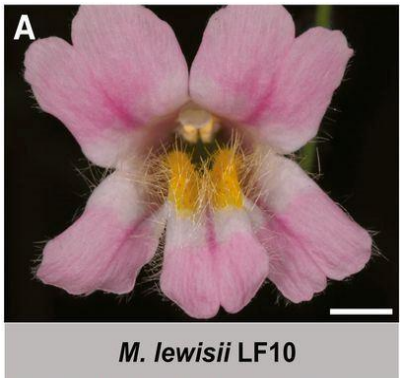

Fig 4: shows the phenotype for the wild type *M. lewisii* LF10. This is the expected phenotype for the mutant flowers that had been infiltrated with EFP.

DISCUSSION

Previous research showed that mutations in the enhancer of flavonoid production gene result in a pale color in Japanese morning glory (Morita et al. 2014). They showed that EFP is likely to act on the flavonoid biosynthetic pathway, especially the earlier enzymes involved in this pathway. We wanted to see if EFP interacts with either CHS or CHI, the enzymes involved in the first two committed steps in the pathway. To test this, we wanted to infiltrate EFP agrobacterial cells with either CHS or CHI to potentially induce an overexpression phenotype in wild type *Mimulus lewisii*.

So far, we have built the entry clone plasmid fragment for *EFP*, *CHI*, and *CHS*. Then, the three entry clones were inserted into their respective pEarleyGate vectors mentioned earlier (Earley et al. 2006). The vectors p201YN and p202YC each have half of the EYFP fluorescence protein. Therefore, we can only see fluorescence if the sequences inserted into the two vectors interact with each other. We were able to successfully sequence the final plasmids for *CHI* p201YN, *CHI* p202YC, *EFP* 201YN, and *EFP* p101. CHS

did not show an insert when we ran a PCR to check for the enzyme after the LR reaction. Unfortunately, when we ran a colony PCR to check for *CHI* p201YN and *CHI* p202YC insertions, they inserted in the opposite orientation. Nevertheless, we continued with the *EFP* p101 and transformed this plasmid and *NEGAN* p100 into *Agrobacterium tumefaciens* before agroinfiltration.

As mentioned before, we infiltrated the agrobacteria of *NEGAN* p100 and *EFP* p101 into the wild type *M. lewisii* LF10. We performed the transient assay on the wild type LF10 to ensure that when we view EFP expression under the confocal microscope, the gene will be under normal conditions. When we viewed the transient assay under the confocal microscope, we found that EFP is mainly localized in the nucleus and the plasma membrane. Therefore, it is possible that EFP could function similar to that of a G protein-coupled receptor to activate signal transduction pathways, since G protein receptors are also localized in the nucleus and plasma membrane.

Prior to the stable transformation, the EMS mutants that showed the white colored phenotype for LF10 had to be tested to see if there was a mutation in the *EFP* gene. Instead of doing a bulk segregant analysis along with deep sequencing (Yuan et al. 2013b) to find mutations, we hypothesized that there was a mutation in the *EFP* gene that caused this phenotype. This was because none of the genes directly involved in the anthocyanin pathway showed mutations and *EFP* was a primary choice for impacting anthocyanin production outside of the pathway. Even though mutations were discovered in the *EFP* gene for these mutants, a rescue experiment was done infiltrating

the *EFP* vector to confirm that *EFP* is the causal gene to have induced the pale-white phenotype.

One hypothesis as to how EFP interacts with CHS or CHI is that they form an enzyme complex and channel intermediates. EFP plays a role in flavonoid production and flavonoids are crucial to plants for various reasons ranging from pathogen resistance to UV protection. Substrate channeling is an efficient and fast way of passing the metabolic product of EFP. Furthermore, it has been shown that EFP related CHI interacts with CHS during the initial steps of the pathway where enzyme complexes are formed (Morita et al. 2014). If this is the case, then it would be likely that CHI related EFP also interacts with CHS in the enzyme complexes.

By learning more about EFP and the mechanism in how it interacts with the flavonoid biosynthetic pathway, we can potentially regulate the amount of flavonoid production in plants. Flavonoids have many diverse roles including protection from UV light, regulators of symbiotic interaction with microorganism, plant pathogen resistance and color pigmentation. However, flavonoids also are beneficial to human health as they are known to reduce free radicals, and inhibit bacterial strains and viral enzymes (Dao et al. 2011). Research has also shown that flavonoids are known to prevent certain types of cancer and cardiovascular diseases (Havsteen 2002). Therefore, by exploring how *EFP* is involved in enhancing flavonoid production, this application can be applied to other types of plants and potentially aid in engineering new pharmaceutical products.

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