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Investigating the Expression of *GROWTH-REGULATING FACTOR 2 (GRF2)* in *Arabidopsis thaliana*

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ABSTRACT

Arabidopsis thaliana is a model organism that is used in laboratories across the world for the study of cellular and molecular processes in plants. *GROWTH-REGULATING FACTOR 2 (GRF2)* is a transcription factor that promotes leaf growth in Arabidopsis. It is a member of a family of transcription factors that is conserved across all land plants. Previous studies have characterized *GRF2* expression in leaves, but *GRF2* expression in floral organs has not been studied as extensively. *GRF2* acts redundantly with other members of the GRF transcription factor family during floral development. *grf1/2/3/5* quadruple mutants show malformed gynoecia, severe fusions between floral organs, and a reduction in the number of stamens and petals. These defects are much more severe than in *grf1/3/5* triple mutants, indicating an important role for *GRF2* in flower development. Here, we created *a pGRF2:GUS* transcriptional reporter to visualize the transcriptional activity of the *GRF2* gene in Arabidopsis flowers. Inflorescences from transformed plants were embedded in wax and sectioned via microtome. Tissues that express the GUS enzyme under the transcriptional control of the *GRF2* promoter are stained blue, allowing us to visualize the transcriptional activity of *GRF2*. We found that *GRF2* is expressed in the inflorescence meristem and broadly in young flowers. The staining is somewhat variable with dark spots of GUS staining visible in some tissue sections. Further work is needed to elucidate the regulatory networks responsible for controlling *GRF2* expression in inflorescences. In particular, additional translational

reporters are being made to investigate whether *GRF2* expression is regulated by MIR396 in flowers, as it is in leaves. This study provides valuable insights into the expression of *GRF2* in Arabidopsis.

BACKGROUND

Flowering plants are incredibly important organisms within Earth's ecosystems and have long fascinated us for their beauty and amazing diversity. *Arabidopsis thaliana* is a small plant in the mustard family that is used as a model organism for basic research in plant biology. The study of molecular processes and the regulatory networks that govern plant development has exploded in recent years. This thesis aims to contribute to the scientific understanding of the molecular regulation of floral development in *Arabidopsis* by characterizing the expression of the gene encoding the transcription factor GROWTH-REGULATING FACTOR 2 (GRF2).

Vascular plants grow through the continuous action of self-regenerative stem cell populations known as meristems. Post-embryonic development begins from stem cells in the center of root apical meristems (RAM) and shoot apical meristems (SAM). The SAM is responsible for the initiation of leaves, stems, and axillary meristems during the vegetative development of flowering plants. Lateral organs are initiated by the SAM: leaves during the vegetative phase of development and flowers during reproductive development. When the plant switches over to the reproductive development phase, the SAM transitions to a dome-shaped inflorescence meristem (IM) (Smyth et al., 1990). The IM initiates floral meristems (FM) as lateral organs instead of leaves. In *Arabidopsis*, FMs arise on the flank of the IM. Floral meristem cells initiate floral organ primordia in four concentric whorls that adopt a fate as either as sepal, petal, stamen, or carpel (Figure 1). Most dicot flowers are composed of these four organ types. In *Arabidopsis*, the

outermost two whorls consist of the sepals, the protective covering of a developing flower, and the petals, which serve to attract pollinators. The innermost two whorls contain the reproductive organs. Stamens, the male reproductive organs that contain pollen, arise in the third whorl. The fourth and innermost whorl contains the female reproductive organs, the carpels.

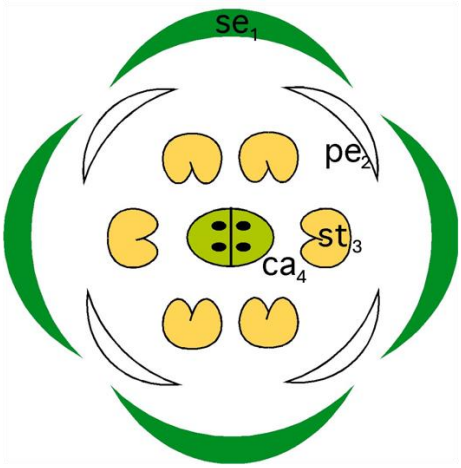

Figure 1: **The wild-type *Arabidopsis thaliana* flower.** The floral organs of *Arabidopsis* grow in four concentric whorls. Sepals (se), petals (pe), stamens (st), and carpels (ca) are shown. Numbers indicate the whorl.

The development of flowers is under the strict sequential control of floral meristem identity genes, floral organ identity genes, and downstream effectors, including noncoding RNAs, enzymes, and transcription factors, which orchestrate the specification of diverse tissues and cell types within each floral organ (Krizek & Fletcher, 2005). The floral meristem identity genes are expressed in floral primordia initiated at the periphery of the IM and ensure that these primordia adopt a flower fate rather than continuing as IM

stem cells (Coen & Meyerowitz, 1991; Krizek & Fletcher, 2005). A second function of the floral meristem identity genes is to activate expression of the floral organ identity genes (Krizek & Fletcher, 2005). Floral organ identity genes encode transcription factors that act in different combinations to specify sepal, petal, stamen, and carpel development (Coen & Meyerowitz, 1991; Krizek & Fletcher, 2005). Genetic analysis of these genes led to the development of the ABCE model for the specification of floral organ identity.

The ABCE model, depicted graphically in Figure 2, explains how distinct combinations of four classes of genes—A, B, C, and E—determine the specific identities of sepals, petals, stamens, and carpels within a flower (Coen & Meyerowitz, 1991; Krizek & Fletcher, 2005). The class A, B, and C genes are each active in two adjacent whorls of the flower with class A genes active in whorls one and two, class B genes active in whorls two and three, and class C genes active in whorls three and four. In contrast, the class E genes (*SEPALLATA1-4*) are active in all four whorls and are redundantly required to specify the identity of all four types of floral organs (Krizek & Fletcher, 2005). The class A genes *APETALA1 (AP1)* and *APETALA2 (AP2)* specify sepal identity in the first whorl. A function combined with the class B genes, *APETALA 3 (AP3)* and *PISTILLATA (PI)*, specify petal identity in the second whorl. Class B function combined with the activity of the class C gene *AGAMOUS (AG)* confers stamen identity in the third whorl, and C function alone specifies the identity of carpels in the fourth whorl (Krizek & Fletcher, 2005). Interestingly, the class A and C genes are mutually antagonistic and are not expressed in the same tissues. Class A activity in whorls one and two prevents the class C gene from being active in the outer two whorls and class C activity in whorls three and

four prevents class A genes from being active in the inner two whorls. The class ABCE genes encode transcription factors with all but the class A gene *AP2* encoding MADS domain transcription factors. AP2 is a member of a different family of transcription factors known as the AP2/ERF family. Downstream targets of these transcription factors, that mediate the growth and elaboration of each type of floral organ, have started to be identified (Ó'Maoiléidigh et al., 2013; Wuest et al., 2012).

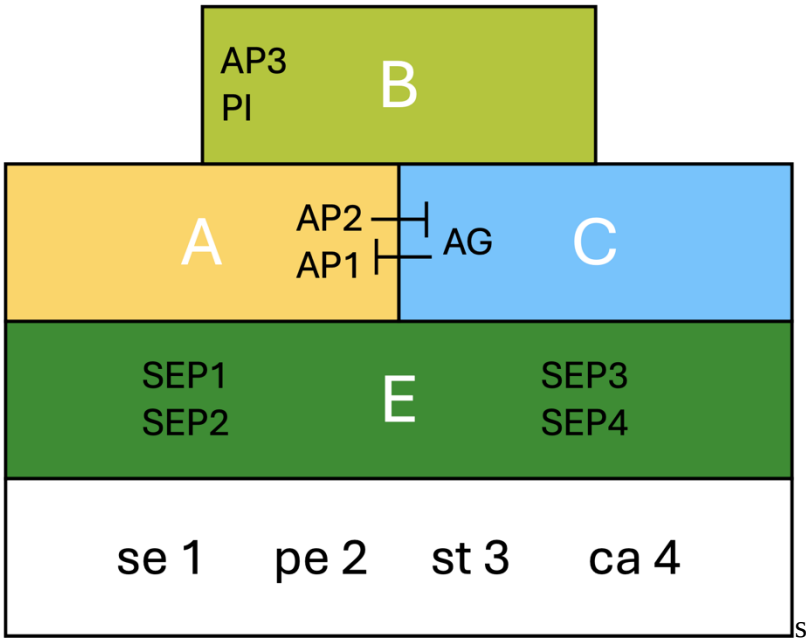

Figure 2: **The ABCE model of floral organ patterning.** Sepal (se) identity is determined by the combined action of class A (*AP1* and *AP2*) and E (*SEP1-4*) genes, while petal (pe) identity emerges through the collaborative function of class A, B (*AP3* and *PI*), and E genes. Stamen (st) identity is governed by class B, C (*AG*) and E genes, whereas carpel (ca) identity is conferred by the function of class C and E genes.

Transcription factors are master regulators of gene expression and they are critical for controlling the developmental timing and spatial expression of genes in eukaryotic organisms. The GROWTH-REGULATING FACTOR (GRF) family is a small family of plant-specific transcription factors first identified in rice almost 25 years ago (Omidbakhshfard et al., 2015; van der Knaap et al., 2000). The GRFs are conserved across all land plants, including mosses, and are known to play a role in a diverse range of developmental processes, including but not limited to floral development, root growth, leaf growth, and oil production in seeds (Omidbakhshfard et al., 2015). The Arabidopsis genome encodes nine GRFs expressed in roots and shoots, particularly in the growing tips of these tissues where cell proliferation occurs suggesting that they function in early stages of growth and development (Horiguchi et al., 2005; Kim et al., 2003). Seven of the nine GRFs (GRF1, 2, 3, 4, 7, 8, 9) in Arabidopsis contain a binding site for the regulatory microRNA miR396 which acts to downregulate their expression in leaves (Rodriguez et al., 2010). GRF expression is high in young leaves and declines as leaves mature (Kim et al., 2003; Omidbakhshfard et al., 2015; Rodriguez et al., 2010). miR396 shows the opposite expression pattern with low expression early in development and higher levels as cell proliferation declines (Rodriguez et al., 2010). The balance between the levels of GRF and miR396 expression regulates cell proliferation within leaves (Rodriguez et al., 2010).

GRF proteins contain highly conserved QLQ and WRC regions at the N-terminus (Kim et al., 2003). The QLQ (glutamine, leucine, glutamine, IPR014978) domain is conserved across all members of the GRF family and plays an important function as a protein-

protein interaction domain (Omidbakhshfard et al., 2015; van der Knaap et al., 2000). This domain facilitates the interaction between GRFs and GRF-INTERACTING FACTOR 1 (GIF1), a transcriptional co-activator that physically associates with GRF proteins, forming a transcriptional complex (Horiguchi et al., 2005; Kim & Kende, 2004). The other conserved region of the GRF protein, the WRC (tryptophan, arginine, cysteine, IPR014977) domain, contains a C3H motif DNA binding motif and several basic amino acids which likely act as a nuclear localization signal (van der Knaap et al., 2000). The WRC regions of GRF1-4 and GRF7-8 also contain a region of near perfect sequence complementarity with miR396 (Liu et al., 2009).

In their study, Kim and Kende (2004) demonstrated the involvement of *GIF1* in the regulation of leaf and petal shape. *gif1* single mutants develop shorter, narrower leaves and petals and display an overlapping phenotype with *grf1/2/3* triple mutants. The similar expression patterns of the *GIF* and *GRF* genes are further evidence that these proteins interact and influence the size and shape of leaves and petals. Furthermore, *35S:MIR396* transgenic plants, in which the MIR396 gene is overexpressed under the control of the cauliflower mosaic virus *35S* promoter, develop narrow leaves and display a similar phenotype to the *gif1* single mutant plants (Liu et al., 2009). These studies highlight the importance of post-transcriptional regulation of the *GRF*s in coordinating both floral and leaf development.

Here we focus our attention on *Arabidopsis GROWTH-REGULATING FACTOR 2*. *GRF2* functions redundantly with *GRF1* and *GRF3* to promote cell proliferation in leaves

(Kim et al., 2003). Single-gene loss-of-function mutants of *GRF1*, *GRF2*, and *GRF3* have a wild-type appearance, but the triple mutant *grf1/2/3* plants develop narrower, smaller leaves and shorter petioles as compared with wild-type plants (Kim et al., 2003). Overexpressing *GRF2* using a *35S:GRF2* construct significantly enhances leaf and cotyledon surface area as compared to wild-type plants, demonstrating that elevated *GRF2* expression effectively promotes leaf growth (Kim et al., 2003). The increase in leaf size is due to an increase in cell number; thus, *GRF2* promotes the growth of leaves by promoting cell proliferation (Horiguchi et al., 2005; Kim et al., 2003; Rodriguez et al., 2010).

Research is advancing in identifying the role of *GRF2* in floral development. Although *grf2* single mutants exhibit no discernible phenotype in floral organs (Figure 3), studying higher-order mutants offers a more comprehensive understanding of *GRF2*'s specific contributions to floral development (Kim et al., 2003). *grf1/2/3/5* quadruple mutants display a pleiotropic phenotype and severe defects in floral organs (Lee et al., 2018). These defects include valveless or single-valved gynoecia (the female reproductive organs), severe fusions between floral organs, and a reduction in the number of petals and stamens. Organ fusion was observed within whorls (sepal-sepal fusion, petal-petal fusion, and stamen-stamen fusion) and between whorls (petal-stamen fusion and stamen-carpel fusion). *grf1/3/5* triple mutants had slight aberrations in floral organ separation and number as well, but the addition of a *grf2* mutation results in a much more severe phenotype, indicating an important role for *GRF2* in regulating floral organ development (Lee et al., 2018). While *GRF2* promotes leaf growth, the available data does not support

a direct role for *GRF2* in promoting floral organ growth, although such a role could be masked by genetic redundancy with other *GRF*s. *GRF2* may play a role in floral meristem growth. The reduced numbers of petals and stamens in the quadruple mutant could result from insufficient floral meristem cells for the initiation of the normal numbers of petals and stamens. Furthermore, the production of mosaic floral organs in *grf1/2/3/5* quadruple mutants suggests a loss of boundaries between floral organ primordia within a whorl and between whorls that may be a related to smaller floral meristem size.

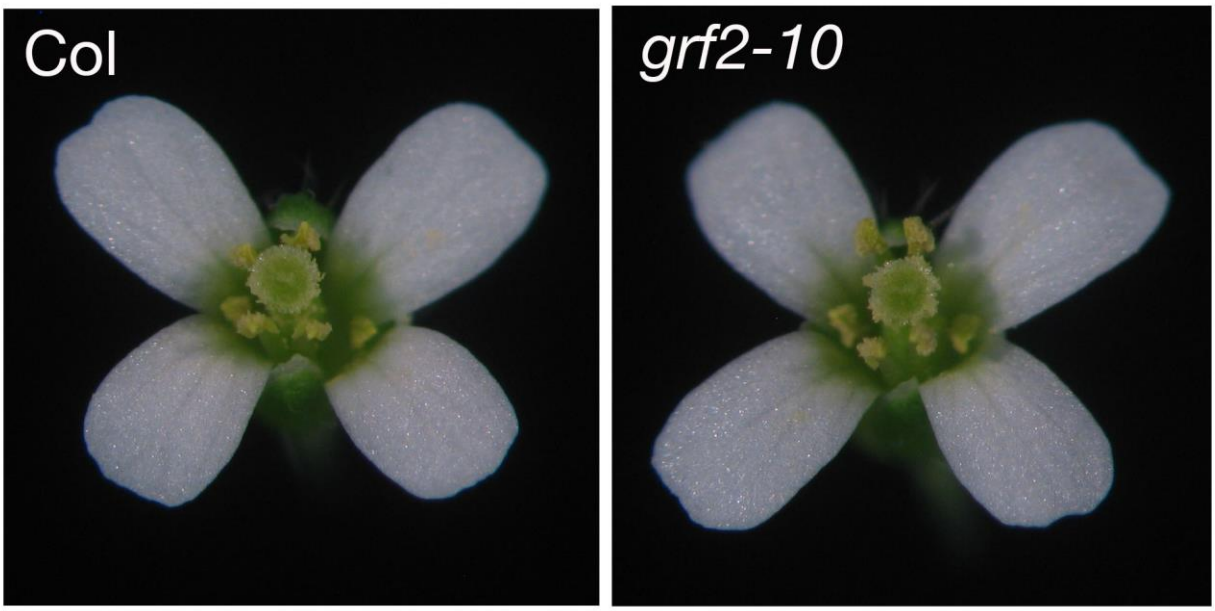

Figure 3: ***grf2* single mutant flower as compared to wild type.** A wild-type Arabidopsis flower from the Columbia background (left) in juxtaposition with the *grf2* single mutant flower (right).

Several studies have examined the expression of *GRF* genes in inflorescences. A 2003 study showed strong expression of *GRF2* in 2-mm-long shoot tips containing the SAM and young flower buds, with expression dropping off significantly in mature flowers (Kim et al., 2003), which is consistent with *GRF2* acting at early stages of flower development. In addition, β-glucuronidase (GUS) reporter constructs have been made previously to explore *GRF2* expression. Kim et. al, 2003 created a *pGRF2:GUS* transcriptional reporter using a promoter of approximately 1.5kb in length. This reporter did not show obvious GUS staining in very young flowers, though there was staining in the sepals and anthers of older flowers, as well as in the style and central region of anthers in older flowers (Kim et al., 2003). Another *GRF2:GUS* transcriptional reporter using a promoter approximately 2kb in length showed GUS staining in the pedicels and pistils of *Arabidopsis* flowers (Liang et al., 2014). This construct appears to show expression of *GRF2* in and around the inflorescence meristem and in the pedicels and pistils of open flowers, though sectioned tissue was not presented. A translational reporter using approximately 2kb of the *GRF2* promoter shows staining throughout young flowers, in the sepals, stamens, and carpels of stage 7 flowers, and throughout the carpels of stage 7-8 flowers. *GRF2* expression drops off in flowers at stage 11 and older (Lee et al., 2018). Together this work suggests that *GRF2* is expressed broadly in young flowers with expression becoming restricted to reproductive organs during development.

Our goal with this work was to gain a more detailed understanding of *GRF2* expression in the IM and developing flowers. We have made a new transcriptional reporter that utilizes 3.8kb of 5' upstream sequence which includes upstream regions bound by known

floral regulators in chromatin immunoprecipitation sequencing (ChIP-Seq) experiments (Figure 4). Previous transcriptional and translation reporters used constructs containing between 1.5-2kb of the promoter region, which do not include these binding sites. The ABCE floral organ identity transcription factors AP1, AP3, PI, and AG, as well the growth regulators AINTEGUMENTA (ANT) and AINTEGUMENTA-LIKE6 (AIL6), have binding sites within the 3.8kb *GRF2* transcriptional reporter described here. If these floral regulators modulate *GRF2* expression, then the 3.8kb promoter will give a more accurate picture of *GRF2* expression than constructs that do not include these binding sites. Additionally, we are now constructing *GRF2* translational reporters using this same 3.8kb promoter fragment to investigate the possible role of MIR396 in regulating *GRF2* expression in inflorescences. For this later study, GUS expression will be measured using translational reporters containing either a wild-type *GRF2* genomic sequence or one in which the MIR396 binding site has been mutated without changing the amino acid sequence of the encoded protein.

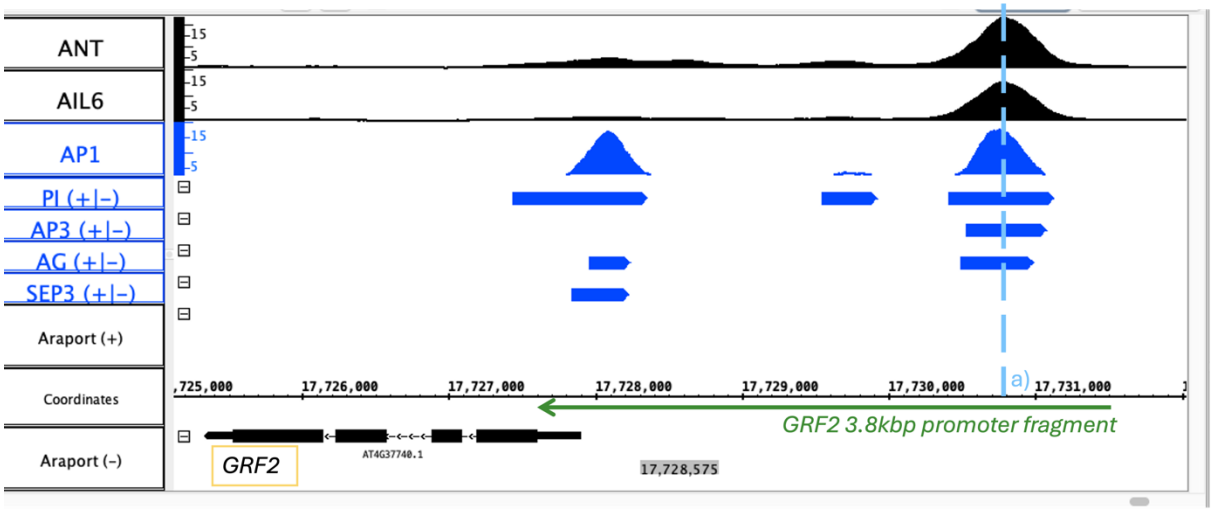

Figure 4: **Chromatin immunoprecipitation sequencing (ChIP-Seq) data of several transcription factors bound to genomic regions upstream of *GRF2*.** The promoter fragment used in our *pGRF2:GUS* construct is shown with the green arrow. *a*) Binding sites for the floral organ identity proteins AP1, PI, AP3, AG, and SEP3 and the growth regulators ANT and AIL6 are included within the 3.8kb upstream region.

RESULTS

pGRF2 Activity Within the Inflorescence

To investigate the spatial expression pattern of *GRF2*, we established a transcriptional reporter system in which the GUS reporter gene is under the control of a 3.8kb *GRF2* promoter fragment. Figure 5 depicts the cloning procedure as described in the Materials and Methods.

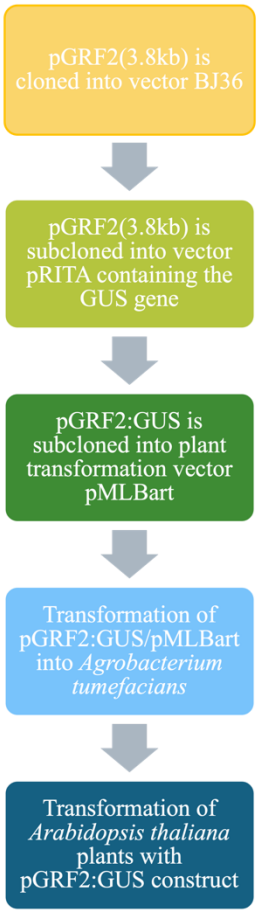

Figure 5: ***Cloning procedure for pGRF2:GUS***. This flowchart depicts the general cloning steps performed for the generation of *pGRF2:GUS* transgenic plants. T1 transgenic plants were confirmed by PCR. T3 homozygous individuals for different lines were GUS stained and the expression pattern for line 20 analyzed in depth.

Examination of GUS-stained whole inflorescences showed blue staining within the center of the inflorescence. The signal is active in the inflorescence meristem (IM) and in surrounding young flowers (Figure 6a). Weak signal is also present in the carpels of older flowers (Figure 6b). Additionally, we observed signal at the base of the pedicels (flower stalks), with the highest intensity noted in the adaxial region.

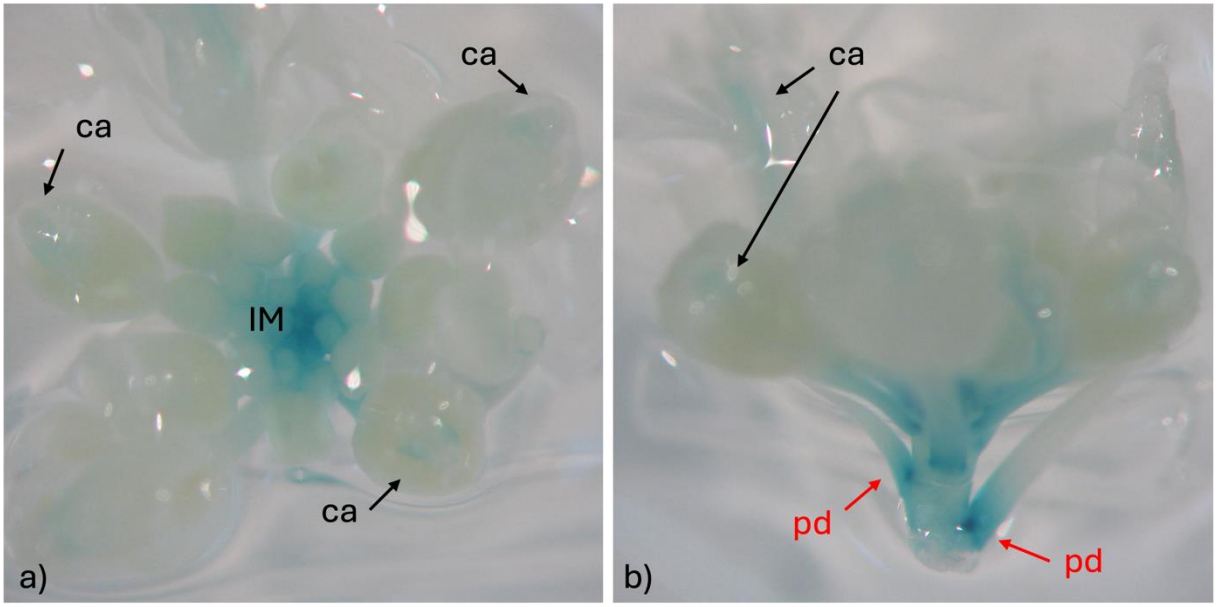

Figure 6: ***pGRF2:GUS whole mount inflorescences.*** a) GUS staining is visible in the center of the inflorescence meristem (IM), in the carpels (ca) of older flowers, and in b) the adaxial axil region of the pedicels (pd).

To gain further insights into the spatial expression pattern within young flower primordia, we sectioned GUS-stained inflorescences. This approach enables the visualization of *pGRF2* expression across various stages of flower development, allowing for a comprehensive assessment of its spatial distribution within the inflorescence.

Figure 7 shows representative examples of sectioned tissues. In sections of the whole inflorescence, the signal is present throughout the IM dome and decreases basally towards the stem (Figure 7A-B). The signal is also present in the pedicels. Interestingly, there are areas of intense staining at the peduncles, indicated by black arrows in Figure 7A-B.

Furthermore, the signal is present throughout young flowers staged 1-2, with minimal variation in expression patterns observed (Figure 7C-E). As flowers progress to stage 3, characterized by the initiation of sepal primordia, the signal intensity increases apically, culminating in the strongest expression within the developing sepals. This expression pattern persisted until stage 6, when the sepals began to cover the flower bud. Notably, a subset of stage 2-5 flowers exhibited intense blue staining at the apex of the floral meristem dome.

The *GRF2* promoter remains active throughout stage 6-7 flowers, with some signal observed in the sepals, petal, stamen, and carpel primordia. At stage 8, there was weak signal persisted throughout the flowers, with stronger expression in carpel primordia. By stage 9 of floral development, there was just faint signal in the carpel. Table 1 summarizes the expression pattern of *GRF2* in each floral stage.

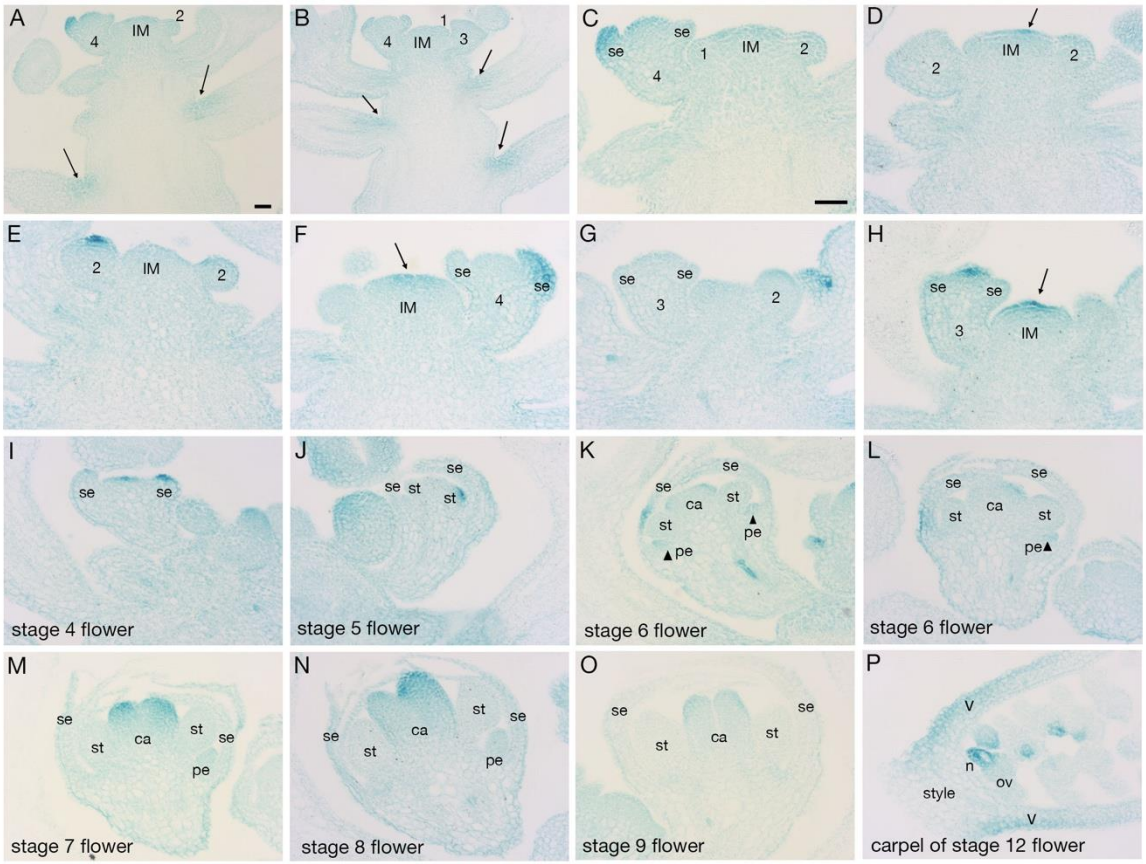

Figure 7: **Composite table showing sectioned *pGRF2:GUS* inflorescences**. a-b) Inflorescences shown at 10X magnification. c-h) Inflorescences shown at 20X magnification i-o) Individual flowers shown at 10X magnification. p) top region of a carpel from a stage 12 flower. Numbers indicate stage of flower development in a-h. IM: inflorescence meristem; se: sepal; st: stamen; ca: carpel; pe: petal; v: ovary valves; n: nucellus; ov: ovule. Black arrows show areas of more intense staining. Size bars show 50μm.

Table 1: ***pGRF2:GUS*** **Expression Pattern in inflorescences**

Stage of development/tissue	Description of stage (based on stages described in Smyth et al., 1990)	GUS expression pattern
Inflorescence meristem and stem below meristem		• Signal throughout IM dome with occasional spots of darker intensity • Signal decreases in the stem below the meristem

		dome with increasing distance from the apex • Signal present in adaxial region where the pedicel meets the stem
Stage 1	St 1: flower buttress arises	• Signal is present throughout floral primordia
Stage 2	St 2: flower primordium forms (distinct from IM)	• Signal is present throughout floral primordia • Occasionally, there is darker staining in the top central region
Stage 3	St 3: sepal primordia arise	• Signal is present throughout sepal primordia and FM
Stage 4	St 4: sepal primordia overlie floral meristem	• Signal is present throughout sepal primordia and FM • Occasionally, there is darker staining in sepal primordia or a spot within the FM
Stage 5	St 5: petal and stamen primordia arise	• Signal present in sepal, petal, and stamen primordia and in central FM dome • Occasionally, there is darker signal in sepals
Stage 6-7	St 6: sepals enclose bud St 7: long stamens stalked at base	• Signal is present in all developing floral organs • Sometimes darker spots of staining are observed within sepals, petals, stamens, or carpels
Stage 8	St 8: locules appear in long stamens	• Signal is strongest in developing carpels
Stage 9	St 9: Petal primordia stalked at the base	• There is light signal in the carpel
Stages 10-11	St 10: petals level with short stamens St 11: stigmatic papillae appear on top of carpel	• No significant staining
Stage 12	St 12: petals level with long stamen	• There is signal in carpel valves and nucellus of ovules

DISCUSSION

GRF2 is expressed throughout the IM and young flower primordia

The GUS staining pattern confirms that the *GRF2* promoter is active at the IM. The *GRF2* promoter is also active in the floral meristem and all floral organ primordia of flowers stage 3-6, but the GUS signal is not uniformly dark throughout the floral meristem or floral organ primordia. Often, there will be a small dot or region of stronger GUS expression. As flowers develop, promoter activity becomes primarily restricted to the carpel. This expression pattern is consistent with *GRF2* functioning within the floral meristem, developing floral organs, and in later stages of carpel development. Previous studies of loss-of-function mutants showed that *grf1/2/3/5* quadruple mutants develop severe fusions of floral organs and a decrease in the number of petals and stamens that develop (Lee et al., 2018). These phenotypes suggest that *GRF2* may play roles in early growth of the floral meristem, floral organ initiation, growth of floral primordia after initiation, and/or boundary establishment/maintenance.

Additionally, *GRF2* appears to be expressed in the reproductive organs of older flowers, specifically in the ovules within the ovaries of the carpel. This corroborates the findings of a translational *GRF2pro:GRF2:GUS* reporter using a promoter region approximately 2kb in length which showed expression in the nucellus of the ovule (Lee et al., 2018). *GRF2* promoter activity in the ovules suggests a possible role in ovule development.

GUS expression in the 3.8kb *pGRF2:GUS* transgenic plants differs from that of transcriptional reporters with shorter promoter fragments

The promoter used in the *pGRF2:GUS* transgenic plants described here is longer than those used in previously published transcriptional or translational *GRF2:GUS* constructs and corresponds to 3.8kb upstream of the start codon of *GRF2*. This promoter fragment includes binding sites identified by ChIP-Seq studies for several floral organ identity transcription factors, such as *API, PI, AP3, AG*, as well as the master floral regulators *ANTEGUMENTA (ANT)* and *ANTEGUMENTA-LIKE 6 (AIL6).* The reporter shows the activity of this promoter fragment, and therefore may be subject to regulation by these transcription factors.

A previous transcriptional *pGRF2:GUS* construct including 1.5kb of the promoter region (the Kim reporter) (Kim et al., 2003) displayed signal within developing anthers of approximately stage 10 and older flowers, the style of the carpel in open flowers, and in the sepals of approximately stage 8-12. This reporter did not have signal at the center of the IM or within younger flowers and thus differs significantly from our results. A second transcriptional reporter that includes a 2kb promoter fragment, the Liang reporter, (Liang et al., 2014) shows signal within the carpels of approximately stage 9-12 flowers, as well as in the pedicels. but GUS signal is not visible in the carpels of open flowers (stage 14 flowers). The expression pattern of our *pGRF2:GUS* construct appears to overlap the expression pattern of the Liang reporter in at least some tissues including the carpels of

stage 9 and 12 flowers and in the pedicels. While our new *pGRF2:GUS* construct showed signal in the carpels of open flowers, this was not observed in the Liang reporter. We also found that many inflorescences display spots of intense staining at the adaxial axis of the pedicels, which is not clearly displayed by the Liang reporter. A detailed comparison of either of these transcriptional reporters and our reporter is not possible given that sectioned material was not presented for either of these earlier reporters.

The differences in the expression pattern suggest that the 3.8kb *GRF2* promoter fragment includes regulatory elements that are important for its expression in the IM and in young flowers that were not included in the two previously published transcriptional reporters. The transcription factors encoded by the floral organ identity genes that bind upstream of *GRF2* could play a role in regulating *GRF2* expression in early stages of flower development. While the class B and C genes are first expressed in stage 3 flowers, the class A gene *AP1* is expressed in stage 1 flowers.

GUS expression in the 3.8kb *pGRF2:GUS* transgenic plants is more similar to that displayed by a *GRF2pro:GRF2:GUS* translational reporter

A *GRF2pro:GRF2:GUS* translational reporter in which a genomic fragment containing 2kb of the promoter region, exons, and introns of *GRF2* was fused to the GUS coding region was developed in order to examine the expression pattern of the *GRF*s in flowers (Lee et al., 2018). Transverse sections of the inflorescence as well as flowers staged 7-12 were presented. The translational reporter shares some similarities with the expression

pattern visualized here. Both reporters show signal within the inflorescence meristem and in young flowers. The *pGRF2(2):GRF2-GUS* reporter shows a decrease in carpel staining from stages 7-9 (Lee et al., 2018). The signal increases in the carpels of stage 10 and 11 flowers before decreasing again. Our *pGRF2(3.8):GUS* reporter also shows variation in the strength of the carpel staining with decreased staining in stage 9, but increased staining at stage 12. Notably, the translational reporter line appears to display stronger signal in the stamens of stage 7 flowers than in our line but less expression in the sepals of stage 7 flowers as compared with our line. The carpels of stage 11 flowers are also stained strongly in the translational reporter while our line does not show signal in the carpels of stage 10 and 11 flowers.

The transcriptional reporter presented here measures the activity of the 3.8kb promoter region. Therefore, the staining pattern reflects the tissues where the *GRF2* gene is actively transcribed. The translational reporter (Lee et al., 2018) fuses the GUS enzyme in frame with a genomic copy of the *GRF2* gene. The expression pattern displayed by this reporter indicates where the *GRF2-GUS* fusion protein accumulates, and is therefore subject to possible post-transcriptional regulation by miR396. miRNAs regulate gene expression by binding to complimentary transcripts and either cleaving the transcript or preventing its translation. The lack of sepal staining in stage 7 flowers of the translational reporter could be due to negative regulation by miR396.

The lack of strong signal within the stamens of stage 7 flowers in our transcriptional reporter could indicate that there are cis-regulatory elements present within the exons

and/or introns of *GRF2* which are present within the translational reporter but not in our transcriptional reporter. Alternatively, negative regulatory elements may be present within our larger 3.8kb promoter fragment.

The pGRF2:GUS Expression Pattern differs from *GRF2* mRNA expression as determined by *in situ* hybridization

In situ hybridization is a powerful molecular tool that allows us to visualize where mRNA accumulates in plant tissues. During this project, *in situ* hybridization of *GRF2* was done previously by Dr. Krizek. Figure 8 shows representative images of *GRF2* mRNA within longitudinal tissue sections. Briefly, *GRF2* mRNA is present throughout the *Arabidopsis* inflorescence and accumulates at the IM dome (Figure 8a), throughout stage 1 and 2 flowers and throughout stage 3 flowers with stronger staining in sepal primordia (Figure 8b-c). However, after stage 4 of flower development, sepal expression decreases and *GRF2* mRNA is detected in petal, stamen and carpel primordia (Figure d-f). This pattern is somewhat different than that visualized by the transcriptional reporter described here with stronger staining in stamen primordia and no staining in the pedicels.

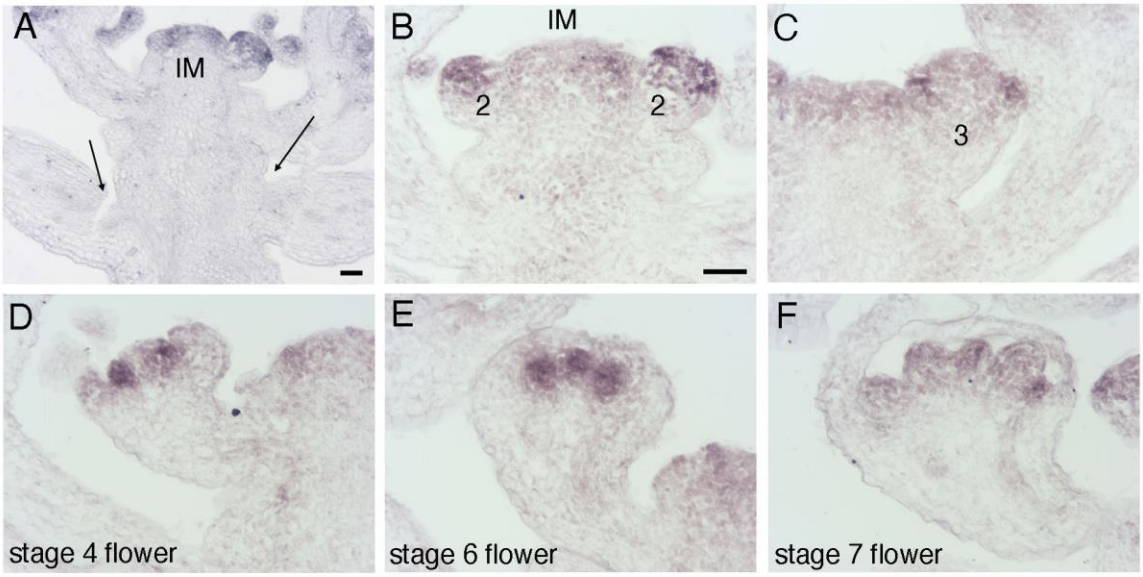

Figure 8: **Localization of *GRF2* expression through *in situ* hybridization.** a) Whole inflorescence at 10X magnification. Black arrows indicate the pedicels. b-f) Flowers depicted at 10X magnification, with floral stages labeled. Size bar indicates 50μm.

Ongoing Research

The transcriptional reporter described here shows the activity of the *GRF2* promoter fused to the coding region of the GUS protein, and as described above there are differences between our 3.8kb reporter with both the *in situ* hybridization data and the GUS translational reporter with 2kb of upstream sequence. Therefore, our reporter does not appear to give an accurate representation of the *GRF2* mRNA accumulation. The differences between the *in situ* data and the GUS reporter presented here could be due to cis-regulatory elements that are missing in the 3.8kb upstream promoter fragment used in our study. For example, there may be a positive cis-regulatory element that promotes expression in developing stamens that is lacking in the 3.8kb 5' sequence. Such an

element may be further upstream, downstream or within the gene itself. Furthermore, any possible regulation by miR396 would not be present within our construct as the miR396 binding site is present within exon 3 of the gene. Thus, the miR396 binding site is present within the 2kb GUS translational reporter. As miR396 negatively regulates GRF expression at the level of transcript accumulation, it seems unlikely that this difference is responsible for the low levels of expression in stamens in our construct.

To investigate the effect of miR396 regulation on *GRF2* expression in flowers, I am developing a translational reporter system in which the GUS coding region is fused directly to the genomic *GRF2* sequence under the control of the 3.8kb promoter region. The activity of the GUS enzyme will reflect any post-transcriptional regulation by miR396. Specifically, we are creating two variants: a genomic *pGRF2:gGRF2-GUS* reporter and a 'miR396 resistant' *grGRF2-GUS* reporter. The miR396 binding site within the pGRF2:*grGRF2:GUS* construct has been mutated to disrupt complementarity between the transcript and miR396 while preserving the amino acid sequence of GRF2.

This system will allow the comparison of the expression pattern of *GRF2* when miR396 can or cannot bind to the transcript. If miR396-mediated downregulation occurs in flowers as it does in leaves, then we will expect the *pGRF2:grGRF2-GUS* reporter will exhibit a much broader expression pattern, as it should not be cleaved by miR396, as compared with the *pGRF2*:*gGRF2:GUS* reporter. If the *pGRF2:gGRF2-GUS* exhibits a similar expression pattern to the *in situ* hybridizations of *GRF2*, then this would suggest that additional cis-regulatory elements are located within the gene.

MATERIALS AND METHODS

Plant Growth Conditions

Plants were grown in a mixture of SunGro Sunshine Mix #1:vermiculite:perlite (8:1:0.3) at 22°C in 16 hours days (100-150μmol m^{-2} s^{-1}).

Construction of *pGRF2:GUS* Transgenic Plants

The *pGRF2:GUS* plasmid was prepared by PCR amplification of a 3.8kb upstream promoter region. Briefly, the 3.8kp *GRF2* promoter was ligated into the cloning vector BJ36 and subcloned into the vector pRITA carrying the GUS coding region. *pGRF2(3.8kb):GUS* was then subcloned into the plant transformation vector pMLBart. *pGRF2:GUS/pMLBart* was transformed into *Agrobacterium tumefaciens ASE* which was then used to transform *Arabidopsis* L*er* plants. T1 transformed plants were selected by spraying with Basta herbicide and confirmed via PCR. 11 lines were obtained and homozygous individuals for each line GUS stained. Line 20 was chosen as a representative line and is described here.

GUS Staining

Inflorescences from approximately 4-week old plants were fixed in 90% cold acetone. The inflorescences were rinsed with a rinse solution (50 mM $NaPO_4$ pH 7.2, 0.5 mM $K_3Fe(CN)_6$, 0.6 mM $K_4Fe(CN)_6$) before being submerged in X-Gluc staining solution

(2mM in rinse solution) (Gold biotechnology). Tissues were then vacuum infiltrated and incubated overnight at 37°C. Chlorophylls were removed via ethanol series (15%, 30%, 50%, 70%, 85%, 95%, 100%, 100%). GUS cleaves the X-gluc substrate to generate a blue precipitate in cells in which the GUS enzyme activity is present. Stained tissues were transferred to xylenes for wax embedding.

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