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# A Mechanism For Sex Determination In Dioecious Cultivated Spinach

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**A MECHANISM FOR SEX DETERMINATION IN DIOECIOUS CULTIVATED SPINACH**

by

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## DEDICATION

To my parents Bruce and Melanie West, for working so hard to provide me with the opportunity to get an advanced education and for supporting me throughout this challenging endeavor. To my brother Kyle West for keeping me humble and helping me relax when my studies got too stressful. I could not have made it this far without your help.

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**TABLE OF CONTENTS**

**LIST OF TABLES**

**LIST OF FIGURES**

## CHAPTER 1: INTRODUCTION

The flowering plants, angiosperms, are a staggeringly diverse group of organisms. Their wide range of morphologies and complex biochemistry has allowed members of this group to spread across the planet into all but the harshest environments. Plants, in many cases angiosperms, have long been critical for human success. Humans have utilized plant material for food, shelter, tools, and medicine. The majority of crops and most natural fibers are produced by angiosperms. Given the incredible diversity of form the characteristics shared across angiosperms are predominantly related to reproduction: including ovules that are enclosed within a carpel, double fertilization leading to the formation of an endosperm, stamens with two pairs of pollen sacs, and features of gametophyte morphology and development. Approximately 85% of angiosperms produce hermaphroditic flowers. This is believed to be the ancestral floral morphology. Plants that have derived floral morphologies can be divided into one of two broad groups, depending on the segregation of reproductive organs. If the female reproductive organ, the gynoecium is separated from the androecium, the male reproductive organs but a single plant produces both types of flowers the plant is said to be monoecious. If the androecium and gynoecium are segregated to individual plants, such that each plant is unisexual and develops flowers with either androecia or gynoecia, they are said to be dioecious. Within each group agronomically important crops can be found, such as the monoecious cucumber and maize, and the dioecious hops and spinach. Although monoecious and dioecious species roughly split the ~12% of angiosperms with unisexual floral morphologies; the groups are not monophyletic and are observed to have evolved independently multiple times (Renner & Ricklefs, 1995). The evolution of dioecy and elucidation of the genetic underpinnings of sex determination is particularly important

for two somewhat interconnected reasons: 1) Compared to sex determination in animals such as *D. melanogaster*, *C. elegans*, and mammals, the sex determining processes in plants are understudied and 2) The recent evolution of dioecious species can give unique insight into the early evolutionary steps required for the development of sex chromosomes, which are so commonly observed in the aforementioned organisms. Understanding how dioecious species evolved from a hermaphroditic ancestor has been an academic endeavor for over a century as Darwin (1877) penned "There is much difficulty in understanding why hermaphrodite plants should ever have been rendered dioecious".

Hermaphroditic flowers allow for the possibility of self-fertilization. This is helpful for sessile plants as it all but guarantees passage of their genes to the next generation however, it has long been known that inbred offspring are often less fit than outbred offspring. This fitness depression, caused by inbreeding, is generally recognized as an important selective force driving the evolution of a dioecious species from a hermaphroditic ancestor (Charlesworth & Charlesworth, 1978a; Charlesworth & Charlesworth, 1978b). In addition to inbreeding depression, a mechanism whereby individual flowers increase their meristemic fitness through specialization is important for the evolution of unisexual flowers from hermaphroditic gender morphs. Such specialization theoretically can result in increased male and female reproductive success, respectively. As such, unisexual flower development via monoecy or dioecy can be considered a reproductive success strategy similar in effect to strategies that regulate the initiation of flowering itself. As a background, I will first briefly review the molecular regulation of inflorescence commitment, followed by the molecular basis of flower development, and finally strategies for the production of unisexual flowers.

**Transition to Flowering**

The sporophyte phase of an angiosperms life cycle can be divided into two growth stages, vegetative and reproductive. During vegetative growth, the plant utilizes the nutrients available to grow and store energy in preparation for the reproductive stage. Precisely timing the transition to flowering is important for sessile plants, as the production and maturation of flowers in many species must occur in a particular time frame and in favorable environmental conditions. Flowering too early or too late could prevent an individual from being able to mate with another of its species or put it into competition with other species it normally avoids. Many plants rely on pollinators to carry gametes to potential mates and improper floral development may interfere or prevent this interaction. Additionally, plants have internal signals that influence transition to flowering including age and nutrient availability which help to ensure proper flower development. In order for the plant to transition to reproductive development a number of signaling pathways that respond to different external and internal conditions must interact synergistically to induce flower development. Expression of *FLOWERING LOCUS T* (*FT*), *FLOWERING LOCUS D* (*FD*), and *SUPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*) are considered the molecular signals that initiate the transition to reproductive development. All of the internal and external signaling pathways ultimately influence the activation or repression of *FT, FD,* and *SOC1.* Once activated, these genes initiate expression of floral meristem identity genes that cause the tissue to develop into a flower.

The major external signals are interpreted by the light-sensing photoperiod pathway and the cold-sensing vernalization pathway. Light is an extremely important external signal and plants have multiple detection mechanisms that inform the plant of the day/night cycle,

type of light (red, far-red, and blue), and intensity of light (Quail, 1998). Plants utilize a five-member family of photoreceptors named *PHYTOCHROME A-E* (*PHYA-E*) (Briggs *et al.*, 2001) that detect and distinguish between red and far-red light, and a two-member photoreceptor family named *CRYPTOCHROME1* and *2* (*CRY1, CRY2*) to detect blue light (Cashmore *et al.*, 1999). The PHY A-E photoreceptors exist in two forms and convert between these forms depending on the light conditions. The Pr (r standing for red) form absorbs red light most efficiently and uses this energy to convert into the Pfr (fr standing for far red) form, while the Pfr form absorbs far-red light and converts to Pr. Sunlight is a mixture of wavelengths including red and far-red light and during the day an equilibrium will develop between the Pr and Pfr configurations. At night the Pfr form will slowly convert back to the Pr form, which is once again ready to detect sunlight. The longer the dark period the more Pfr is converted back to Pr and in this manner the plant is able to determine length of night and day. Long day plants are triggered to flower when the Pr:Pfr ratio is skewed towards Pfr; this is not because of an abundance of light but rather reduced time in darkness which limits the amount of Pfr that decays to Pr (Sharrock & Clack, 2002). The photoperiod cycling of the phytochromes influences members of the PSEUDO-RESPONSE REGULATOR (PRR) family of transcription factors. PRR1 (aka Timing of CAB Expression 1 or TOC1), PRR3, 5, 7, and 9 play a central role in maintaining the circadian clock which influences the expression of ~90% of the *Arabidopsis* transcriptome (Michael *et al.*, 2008). Two genes of particular interest with regard to the transition to flowering are *CONSTANS* (*CO*) (Koornneef *et al.*, 1991; Putterill *et al.*, 1995) and *GIGANTEA* (*GI*) (Fowler *et al.*, 1999), which are the main transcription factors in the photoperiod and circadian clock pathways respectively.

Throughout the day expression of *CO* mRNA slowly increases but is repressed by the transcription factors CYCLING DOF FACTORs (CDF) (Imaizumi *et al.*, 2005) and DAY NEUTRAL FLOWERING (DNF) (Morris *et al.*, 2010). However, during the night its expression is promoted by MULTICOPY SUPRESSOR OF IRA 1 (MSI1) (Hennig *et al.*, 2003) a chromatin remodeling enzyme and the transcription factor FLOWERING BHLHs (FBH) (Ito *et al.*, 2012) leading to a nightly spike of *CO* mRNA. The production of CO protein does not cycle in the same manner as its mRNA due to the daytime repression mediated by PHYB and HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 1 (HOS1) (Lazaro *et al.*, 2015) and the nighttime repression mediated by CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) (Jang *et al.*, 2008) and SUPPRESSOR OF PHYA-105 (SPA) (Laubinger *et al.*, 2006). During short days the CO protein is not translated at significant levels even though the mRNA increases reaching a night time maximum. In long day plants such as *Arabidopsis* and *Spinacia*, functional CO protein must accumulate to a threshold level in order to initiate a transition to flowering. To accomplish this the aforementioned repressors must be nullified. Under long day conditions CDF mediated repression of *CO* mRNA is removed by GI from the circadian clock pathway resulting in elevated levels of the *CO* mRNA during the extended daytime hours. Additionally, GI can stabilize the CO protein. The inhibitors of CO protein function PHYB and COP1/SPA are removed by PHYTOCHROME-DEPENDENT LATE-FLOWERING (PHL) and CRY1/2 respectively (Mockler *et al.*, 2003; Liu *et al.*, 2008). PHL has been shown to interact directly with PHYB-CO protein complex and help prevent PHYB mediated destabilization of the CO protein (Endo *et al.*, 2013). In response to blue-light perception that is characteristic of long day ambient light, CRY1 and CRY2 repress the COP1/SPA complex which allows for an evening accumulation of CO protein. The expression of CO acts

as the main signal integrator for the photoperiod pathway and is influenced by the circadian pathway in a GI dependent manner. Once CO protein levels reach a threshold CO acts as a transcription factor that promotes the expression of FT, a potent transition to reproduction signal (Simon *et al.*, 1996; Samach *et al.*, 2000).

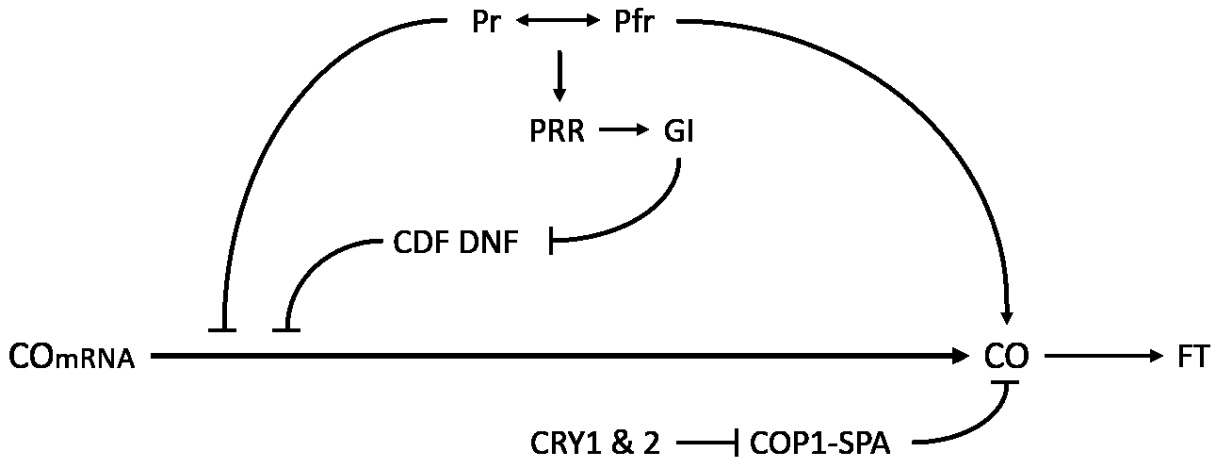


***Figure 1.1*** *– Photoperiod pathway to flowering overview. CONSTANS (CO) mRNA slowly increases throughout the day but is inhibited by PHYTOCHROME red (Pr), CYCLING DOF FACTORs (CDF), and DAY NEUTRAL FLOWERING (DNF). As the day proceeds Pr is converted to the far-red version, Pfr which triggers GIGANTIA (GI) in the circadian clock pathway. The presence of Pfr and GI help remove the inhibition of COmRNA production. CRYPTOCHROME 1 and 2 (CRY1 & 2) help CO protein accumulate in long day scenarios by removing the inhibitors CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) and SUPPRESSOR OF PHYA-105 (SPA). Pfr is also involved in stabilization of CO protein which then activates the expression of FLOWERING LOCUS T (FT).*

The plant's ability to detect light mainly resides in the leaves and indeed this is where CO is translated and produced. CO has been shown to be recruited to the *FT* promoter and initiate transcription, which is then followed by translation of *FT* mRNA within the leaf. For induction of flowering to occur the shoot apical meristem (SAM) must receive signaling to

initiate the switch in developmental programing. As FT is a major contributor to this shift in development and it is produced in the leaves, it must be transported from the leaf to the SAM and it has been observed that FT travels through the phloem (Corbesier *et al.*, 2007). Once FT is trans-located to the SAM it can form a heteroduplex with FD and activate expression of SOC1 which then activates genes that give inflorescence meristem identity to the tissue (Abe *et al.*, 2005; Yoo *et al.*, 2005).

SOC1 is a transcription activator that is critical in flowering time control. SOC1 is observed to integrate pro-flowering signals from the photoperiod pathway briefly described above and also from the vernalization pathway. Vernalization is a period of prolonged cold that is required by some plants for flowering. In such plants, the individual is unable to flower or is delayed in doing so without a sustained drop in temperature. Vernalization prevents a plant from flowering during winter but allows a transition to flower in the following spring or summer. The vernalization pathway controls the expression of *FLOWERING LOCUS C (FLC)*, which is a repressive transcription factor (Michaels & Amasino, 1999). FLC expression is promoted by the FRIGIDIA complex and other complexes through the acetylation and methylation of histones associated with the *FLC* locus allowing *FLC* mRNA to be generated (Michaels & Amasino, 2001; Jiang *et al.*, 2009). When present, FLC prevents the expression of *FT* mRNA in the leaves and SOC1 activity in the SAM thus preventing the transition to flowering (Helliwell *et al.*, 2006). Cold temperatures repress the expression of FRIGIDA and promote the expression of PRC and HDAC complexes that modify the histones of the *FLC* locus and repress transcription (Michaels & Amasino, 1999; Sheldon *et al.*, 1999). Cold temperatures also stimulate the expression of *COOLAIR* and *COLDAIR* which represses *FLC* (Swiezewski et al., 2009; Heo & Sung, 2011) By repressing *FLC* mRNA

production a period of cold temperatures removes one of the inhibitors of *FT* expression and SOC1 action, which creates a permissive genetic environment for the transition to flowering.

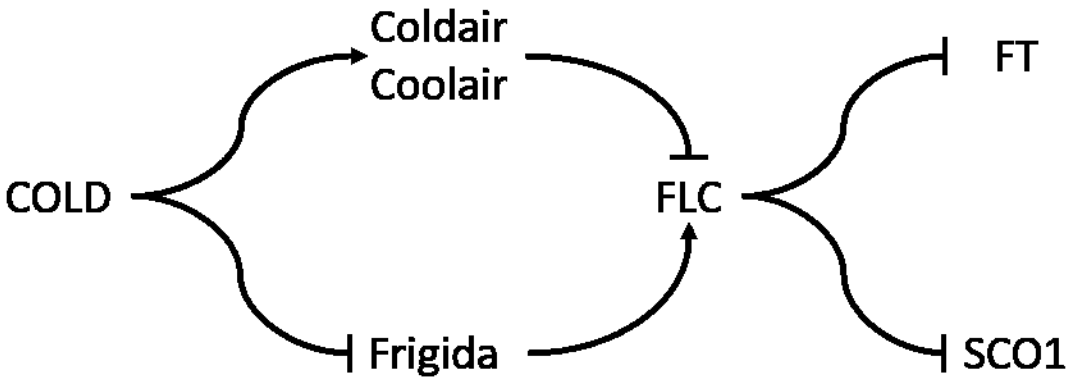


***Figure 1.2*** *– Simplified vernalization pathway. The expression of FLOWERING LOCUS T (FT) and SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1) is repressed by FLOWERING LOCUS C (FLC) which must be removed before a transition to flowering can occur. Cold ambient temperature is able to repress FLC expression by inhibiting the expression of FRIGIDA which activates FLC. Cool temperatures also stimulate the expression of COLDAIR and COOLAIR which repress FLC.*

In addition to the aforementioned flowering pathways it has been observed that phytohormones and gibberellic acid (GA) in particular are able to promote a transition to flowering in *A. thaliana*. The GA hormone is produced through a series of chemical reactions catalyzed by GAoxidases producing the biologically active species $GA_4$. The GA signal is perceived by the soluble GID1 receptor. Binding GA causes a conformational change allowing the receptor to bind regulators of the GA response, the DELLA family of transcription factors (Yamaguchi, 2008). A subset of the GRAS domain transcription factors, the DELLAs have a conserved 17 amino acid DELLA motif near the N-terminal of the polypeptide (Willige *et al.*, 2007). Instead of activating the expression of GA response genes DELLA transcription factors typically repress their targets by binding transcription activators of GA response

genes preventing them from initiating expression. The GA-GID1-DELLA complex allows interaction with the F-box component of an E3 ubiquitin ligase complex (Fu *et al.*, 2004). When both complexes interact, the DELLA transcription factor is poly-ubiquitinated at the DELLA residues. Once poly-ubiquitinated the DELLA protein is targeted for degradation by the 26S proteasome (Fu *et al.*, 2002; Dill *et al.*, 2004). This removes the inhibition of the GA response genes. Distortion of this DELLA domain prevents poly-ubiquitination and is the basis for many GA insensitive DELLA family mutations.

One such target of DELLA repression is PIF4 (the gene product of *PHYTOCHROME INTERACTING FACTOR 4)* a transcription factor involved in thermosensory activation of flowering (Kumar *et al.*, 2012). PIF4 is produced in the leaves and under elevated temperatures is observed to promote the transcription of FT leading to transition to flowering. In *A. thaliana* PIF4 and DELLA were observed to interact physically which blocked PIF4s ability to bind DNA targets (De Lucas *et al.*, 2008). Degradation of DELLA proteins allowed PIF4 to interact with DNA promoters of response genes. Within the SAM, DELLA proteins were observed to interact physically with SQUAMOSA PROMOTER BINDING-LIKE (SPL) transcription factors which serve as an endogenous cue for the transition to flowering (Galvão *et al.*, 2012; Yu *et al.*, 2012). As the plant matures SPLs accumulate in the SAM and promote the expression of SOC1 helping to initiate the shift to reproductive development. SOC1 in turn promotes SPL expression and a feed forward regulation loop is established (Jung *et al.*, 2012). The physical interaction between SPLs and DELLAs where observed to prevent SPLs ability to activate the expression of SOC1 (Yu *et al.*, 2012).

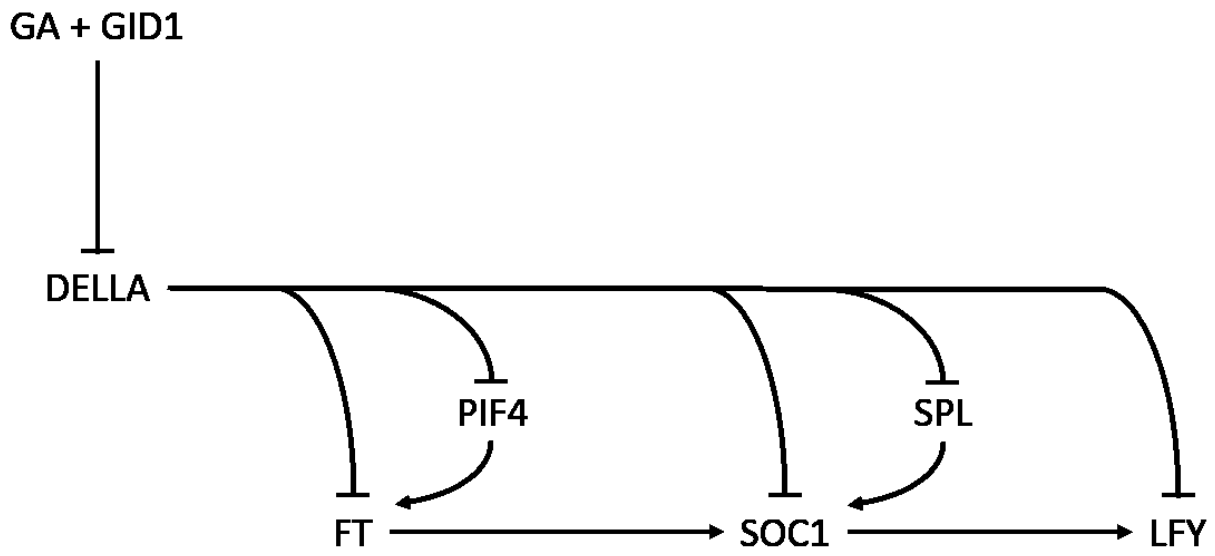


***Figure 1.3*** *– Influence of gibberellic acid and the DELLA transcription factors on flowering. DELLA transcription factors are understood to inhibit the expression of PHYTOCHROME INTERACTING FACTOR 4 (PIF4) and SQUAMOSA PROMOTER BINDING-LIKE (SPL) which are upstream activators of FLOWERING LOCUS T (FT) and SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1) respectively (de Lucas et al 2008, Yu et al 2012). DELLAs also inhibit the flowering signal integrators FT, SOC1, and LEAFY (LFY). This inhibition is removed when the phytohormone gibberellic acid binds its receptor GIBBERELIN INSENSITIVE DWARF 1 (GID1) which causes the degradation of DELLAs.*

The main downstream target of SOC1 is LEAFY (LFY), a transcription factor that is necessary and sufficient for floral development (Schultz & Haughn, 1991). Under short day ambient light LFY expression in the inflorescence primordia is low and unable to trigger a transition to flower development. Long day ambient light stabilizes CO which activates FT that travels from the leaves to the SAM to activate SOC1 which in turn promotes LFY expression and increases LFY abundance past the threshold required for transition to flowering. For this transition to occur the indeterminate growth of the meristem must be inhibited and the floral identity genes must be activated. LFY is able to accomplish both of these requirements. Indeterminate growth of the inflorescence meristem is maintained by

the expression of TERMINAL FLOWER 1 (TFL1) a transcription factor from the phosphatidylethanolamine-binding protein (PEBP) family that also prevents the expression of LFY and APETALA 1 (AP1). AP1 is a MADS box transcription factor that is activated by LFY and important for transition to flowering. Once LFY expression accumulates beyond the flowering threshold it promotes AP1 expression and both AP1 and LFY repress TFL1 causing a switch to determinate growth and endowing the tissue with floral identity. From observations in *lfy* mutants in *Arabidopsis,* it was shown that AP1 is able to initiate a transition to flowering independent of LFY signaling. However, flowering was delayed significantly. *ap1* and *lfy* double mutants were observed to almost entirely lack flowers indicating a synergistic interaction between LFY and AP1 is required for proper flowering control. LFY expression in turn activates a number of key transcription factors that give organ identity to the primordial flower tissue.

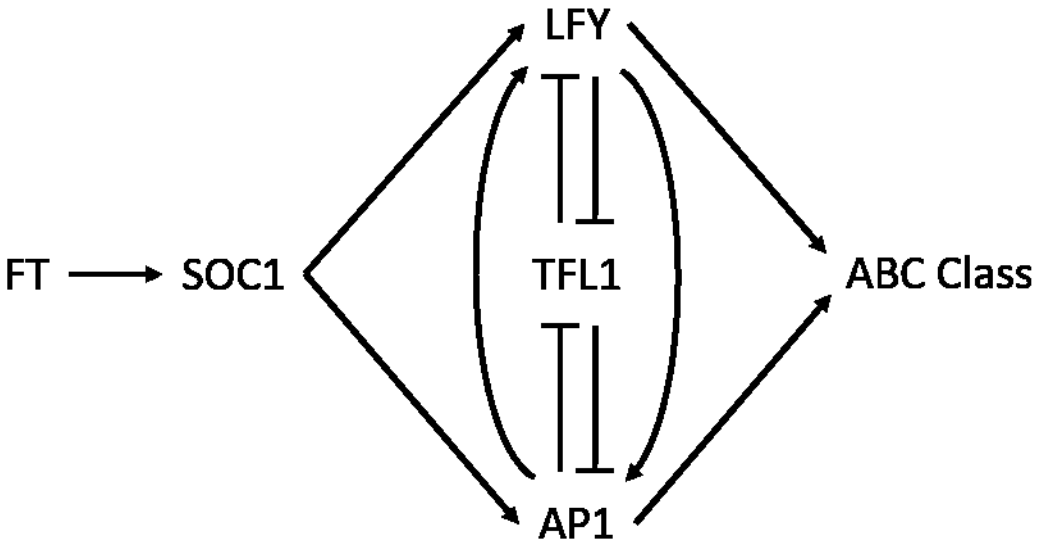


***Figure 1.4*** *– Interaction of flowering signal integrators. Once FLOWERING LOCUS T (FT) is transported to the apical meristem it can activate the expression of SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1) which in turn activates LEAFY (LFY) and APETELLA 1 (AP1). The expression of both LFY and AP1 is normally repressed by TERMINAL FLOWER 1 (TFL1) however, once initiated LFY*

*and AP1 are able to initiate each others expression as well as suppress TLF1. LFY and AP1 are understood to activate the expression of ABC-class genes synergistically although LFY is the main ABC-class expression initiator*.

**Genetics of Flower Development**

The hermaphroditic angiosperm flower is comprised of four organs: the sepals, petals, stamen, and pistil. These organs are produced from non-overlapping concentric whorls of tissue in the flower primordia. The outermost, or first whorl, produces sepals which are leaf-like structures that surround and protect the flower. The second whorl of tissue will produce the angiosperm petals. The most central whorls, the third and fourth, produce the reproductive organs. Stamens, the male reproductive organs, develop in the third whorl of tissue. Female reproductive organs, the pistils, are produced from the fourth whorl in the center of the flower.

Developed by Coen and Meyerowitz in the model organism *Arabidopsis thaliana*, the ABC model describes the genes responsible for flower organ identity (Coen & Meyerowitz, 1991; Pelaz *et al.*, 2000) and later expanded to the ABCE model (Honma & Goto, 2001; Pelaz *et al.*, 2001). Combinatorial expression of genes from the different classes (A through E) result in developing different floral organs. All of the genes, with the exception of *AP2*, are members of the MADS-box family of transcription factors and contain a number of conserved domains. The MADS-box involved in floral organ identification are Type II MADS proteins which share the MIKC domain structure (Ma *et al.*, 1991). The N-terminal MADS domain (M) is followed by the Intervening (I), Keratin-like (K), and C-terminal (C) domains. The MADS domain is highly conserved and is required for protein-DNA interactions, the intervening domain is less conserved and distances the DNA binding domain from the others. The

Keratin-like domain is well conserved and is required for protein-protein interactions while the C-terminal domain is highly variable and Is required for the protein to achieve its specific function. In *Arabidopsis*, *APETALLA 1* (*AP1*) is the A-class organ identity gene. The B-class genes are *APETALLA 3* (*AP3*) and *PISTALLA* (*PI*). There is only one C-class gene, *AGAMOUS* (*AG*). There are four E-class genes *SEPALLATA 1-4* (*SEP1-4*) (Krizek & Fletcher, 2005; Smaczniak *et al.*, 2012).

All of these floral organ identity genes are targets of LFY activation and interact with each other to create specific domains of expression with sharp boundaries that determine which organs develop in which whorl (Busch *et al.*, 1999). The E-class genes are unique as they are expressed in all whorls of the developing flower primordia unlike the other MADS-box genes whose expression is restricted in some manner. SEP3 was observed to produce the most severe phenotype when mutated and is considered to be the most critical of the four (Pelaz *et al.*, 2000; Pelaz *et al.*, 2001). Additionally, the SEP3 protein was observed to interact physically with other SEP proteins as well as with each of the A- through C-class proteins forming tetramers. Expression of AP1 and AP2 are restricted to the first and second whorls. In the first whorl AP1 and SEP3 form a tetrameric complex and canalize the tissue to become sepals. AP3 and PI expressions are restricted to the second and third whorl. Within the second whorl, E-class, A-class and B-class expression overlap and this combination signals for petal organ identity. AG expression is observed in the third and fourth whorls. In the third whorl there is an overlap of E-class, B-class and C-class expression that grants stamen identity to this whorl. Within the fourth whorl, of the floral organ identity genes, only C-class and E-class gene expressions are observed. The C-class and E-class combinatorial expression confers carpel identity to the tissue. The A-class and C-class genes

act antagonistically and inhibit each other's expression creating a sharp, non-overlaping boundry in *Arabidopsis (Irish, 2010)*.

The function of the ABCE floral organ identity genes as well as the type of organ that results from the combinatorial interactions are well conserved in the angiosperms so far investigated. However, not all angiosperms adhere to the expression pattern of the ABCE genes identified in *Arabidopsis*. The tulip (*Tulipa gesneriana*) and lily (*Lilium regale*) flowers develop petal-like organs in the first and second whorl and do not develop sepals or sepal-like organs (van Tunen *et al.*, 1993; Winter *et al.*, 2002; Otani *et al.*, 2016). The third and fourth whorls develop stamens and carpels respectively, as predicted by the quartet model. It was observed that the expression of B-class genes had expanded into the first whorl of the tulip and lily (Kanno *et al.*, 2003) and the combination of A-class and B-class gene activity in the first whorl is responsible for the extra petaloid organs. Another example of altered expression comes from observations in sorrel (*Rumex acetosa*) which develops sepals in the first two whorls, stamens in the third and carpels in the fourth (Ainsworth *et al.*, 1995). Contrary to the expansion of B-class expression in tulip and lily the B-class expression in sorrel was observed to be limited to the third whorl (Ainsworth *et al.*, 1995). These observations lead to the development of the 'shifting boundary' model of flower organ development that hypothesizes that much of the observed floral diversity can be attributed to outward or inward shifts in B-class floral organ identity gene expression (Bowman, 1997; Albert *et al.*, 1998; Theissen *et al.*, 2000; Kramer *et al.*, 2003).

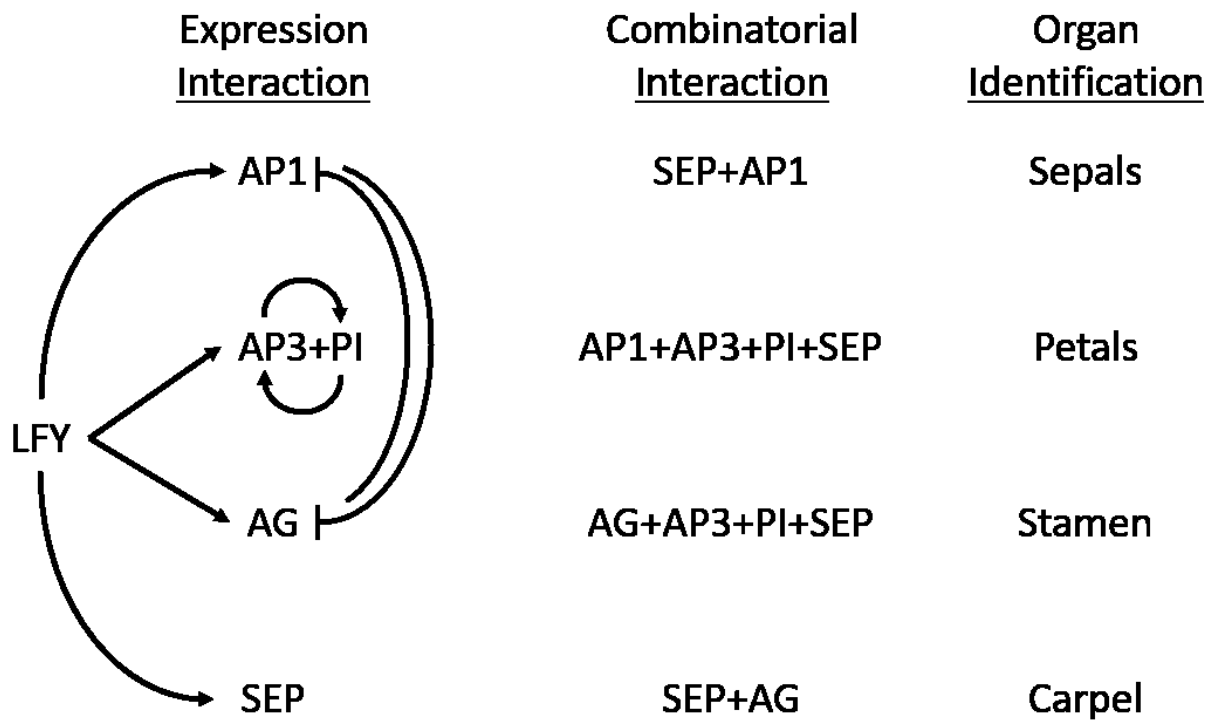


***Figure 1.5*** *– Overview of the initiation, interactions, and morphological result of ABC-class gene expression. The floral integrator LEAFY (LFY) initiates the expression of APETALA 1 (AP1), APETALA 3 (AP3), PISTILLATA (PI), AGAMOUS (AG), and SEPALLATA (SEP). AP3 and PI are able to initiate each others expression which AP1 and AG inhibit each other. These transcription factors interact physically to form heteroquartets, the members of the quartet determine the organ produced from the tissue in which the quartet is expressed.*

**Hypothesized Evolution of Dioecy**

The landmark 1978 Charlesworth and Charlesworth paper characterized the fitness requirements of mutations predicted to lead from an ancestral hermaphroditic population to a dioecious one. The paper suggests that an initial recessive mutation occurs within the population and individuals homozygous for this mutation fail to develop male organs/gametes leading to an individual that produces flowers with only the female function. As long as the obligate females in the population are fit enough to compete with

the hermaphrodites this population would be expected to maintain the male sterilizing recessive mutation. Next, a dominant mutation is predicted to occur that suppresses the development of female organs or gametes leading to individuals that produce only male flowers. Again, if this mutation is not deleterious and the male individuals are able to compete with the hermaphrodites of the population this mutation is expected to persist. Given the mutations described the population would contain: hermaphrodites that do not express the phenotype of either mutation; males that possess the dominate, female sterilizing mutation; females that possess the recessive male sterilizing mutations; and neuters which possess both the male sterilizing and female sterilizing mutations. To avoid the generation of sterile individuals, the mutations responsible for unisexuality must become linked and recombination between these two mutations must be suppressed. Due to the suppression of recombination the region of the chromosome that contains the unisexual mutations would be expected to accrue additional mutations creating unique non-homologous segments that could expand and eventually generate cytologically heteromorphic sex chromosomes.

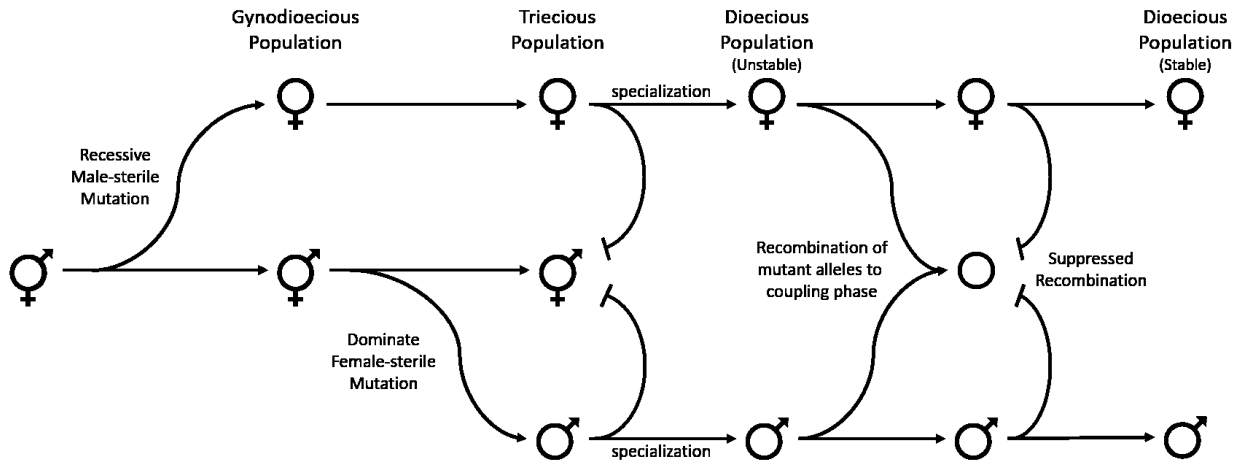


***Figure 1.6*** *– Evolution of dioecy from hermaphroditic ancestor. Initially a recessive, male sterilizing mutation arises in a hermaphroditic species. If the feminized mutant can compete with the*

*hermaphrodite morph a gynodioecious population emerges. Next a dominate female sterilizing mutation is predicted to arise producing a triecious population if the resulting male individual can compete with the hermaphrodite. The unisexual morphs could then outcompete the hermaphrodite by specializing in producing only one of the two sexes. Recombination between the unisexual mutations can produce a sterile individual to avoid this outcome recombination between the mutations must be suppressed. Once this is accomplished a dioecious population is the result.*

Following approximately 40 years of scientific scrutiny, three major concerns with this model and its predictions have been identified. The model ultimately predicts the generation of cytologically distinct sex chromosomes, yet there are few examples of dioecious plants that have morphologically unique sex chromosomes (Charlesworth, 2002). The sterilizing mutations that impart sex are assumed to be independent, however, observation of floral development in dioecious species revealed that abortion of male or female organs typically occurs at the same developmental stage. While this is not in contradiction of the theory, it does suggest that the mutations do not act independent of each other (Diggle, Pamela K *et al.*, 2011). The model's initial feminizing mutation would produce a gynodioecious population consisting of hermaphrodites and female individuals and predicts the evolution of a dioecious species from this sub-dioecious population. However, dioecious species are most often observed to have evolved from monoecious ancestors (Renner & Ricklefs, 1995). Research aimed at addressing these weaknesses and adapting the theory for the evolution of dioecy continues to this day.

Evolution of dioecy does not need to originate directly from a hermaphroditic ancestor and an alternative hypothesis suggesting evolution from a monoecious ancestor, through a paradioecious intermediary, and finally establishing a dioecious population was developed during the 1970's and 80's (Lloyd, DG, 1975; Lloyd, 1980a; Webb, 1999; Renner & Won,

2001). An advantage of this model is that monoecious species already possess gynoecium and androecium sterility mutations that are maintained in the population. This avoids the generation and invasion of andro/gynoecium sterility mutations into a hermaphroditic population. Additionally, monoecious species are not observed to have cytologically distinct sex chromosomes to harbor mutations for unisexualitiy but rather have evolved regulatory mechanisms to ensure coordinated expression of the sterility genes to avoid the production of sterile flowers. Transition to paradioecy may then occur with gender specialization that skews the ratios of male and female flowers produced on an individual (Lloyd, D, 1975). The two most likely selective forces involved in such a transition from a monoecious population are increased seed fitness as a result of an increased ratio of female to male flowers and a reduced rate of self-fertilization (Charlesworth & Charlesworth, 1978b). Accumulation of gender specialization mutations would lead to a population of inconsistent males and females. Individuals with inconsistent sex produce a majority of flowers displaying one sexual morph but a few flowers of the opposite gender. Inconsistent females are not expected to be common as the few male flowers produced by an otherwise female individual are able to self-fertilize most if not all of the gynoecia (Charlesworth & Charlesworth, 1978b). Thus, inconsistent females not only negate the advantages of outcrossing but also incur a reduction in fitness due to inbreeding depression. However, inconsistent males are less disadvantageous as maturing fruit produced from self-fertilization is less injurious so long as developing the supporting structures requires little energy allocation. The resulting paradioecious population would be expected to include females and inconsistent males. At this point any mutation(s) that removes the inconsistency of the male would thus render the population dioecious.

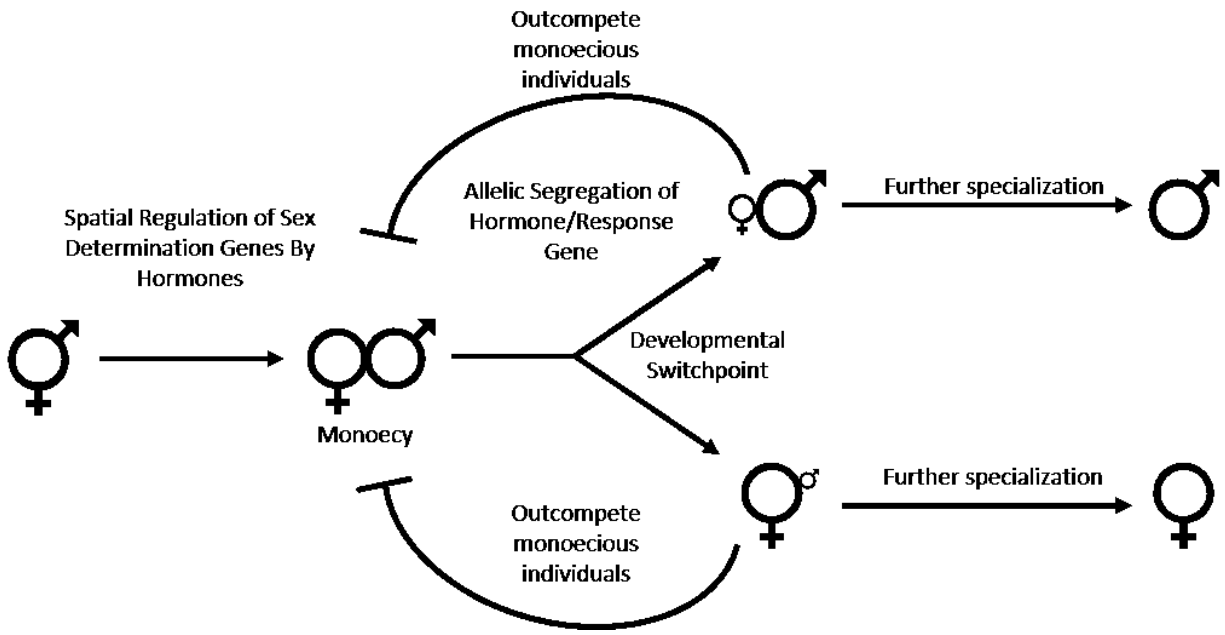


***Figure 1.7*** *– Evolution of dioecy from a monoecious ancestor. Monoecy depicted as joined male and female icons, inconsistent males and females represented by regular sized symbol with one of reduced size attached. Given a monecious species, mutations that enhance male and female reproductive success would be expected to be selected for resulting in a population of inconsistent males, monoecious individuals, and inconsistent females. It is suggested that inconsistent females would be rare, as a single male flower on an otherwise female individual could fertilize the majority of gynoecium thus reducing its fitness. The females and inconsistent males are expected to continue to specialize and outcompete monoecious individuals at which point any mutation that removes the inconsistency would render the species dioecious.*

Early work investigating the unisexual development in monoecious and dioecious species found that the application of phytohormones was sufficient to cause a change in the sexual characteristics of treated flowers. Although patterns can be seen, no hormone has been observed to be purely masculinizing or feminizing. Gibberellic acid is typically masculinizing as observed in *Solanum carolinense, Asparagus officinalis,* and *Cannabis sativus*

(Atal, 1959; Amruthavalli, 1978; Lazarte & Garrison, 1980) but has a feminizing effect in *Luffa acutangula*, *Hyoscyamus niger*, and *Zea mays* (Resende & Viana, 1959; Bose & Nitsch, 1970; Hansen *et al.*, 1976b). Auxin has a feminizing effect in *Cannabis sativus*, *Silene pendula*, and *Cucumis* sp. (Heslop-Harrison, 1956; Heslop-Harrison & Heslop-Harrison, 1958; Chailakhyan, MK & Khryanin, V, 1978b; Malepszy & Niemirowicz-Szczytt, 1991), but is masculinizing in *Mercurialis annua* and *Cleome spinosa* (De Jong & Bruinsma, 1974; Hamdi *et al.*, 1987; Durand & Durand, 1991). Ethylene has been studied extensively in curcubits and found to be feminizing (Atsmon & Tabbak, 1979; Yin & Quinn, 1992; Yin & Quinn, 1995; Trebitsh *et al.*, 1997; Kahana *et al.*, 1999; Krupnick *et al.*, 1999; Mibus & Tatlioglu, 2004; Boualem *et al.*, 2008; Martin *et al.*, 2009) except for watermelon in which ethylene is masculinizing (Rudich, 1990). Given this background, the mutation of a single gene controlling the expression of the pre-existing endogenous signaling mechanism of sexual determination would be sufficient to establish dioecy (Renner, 2016). If this gene was located near the centromere where recombination is naturally suppressed, a single mutational event could render a dioecious species from a monoecious population. This scenario may not be as far-fetched as it initially appears, for example in maize recombination is restricted to the ends of chromosomes (Rodgers-Melnick *et al.*, 2015) and the recombinationally suppressed regions may include a large proportion of genes as in barley where ~20% of genes are found in non-recombining regions (Baker *et al.*, 2014).

**Dioecious Spinach**

Cultivated spinach, *Spinacia oleracea* is found within the Chenopodiacea, a subfamily of the Amaranthaceae family. Recent efforts to elucidate the phylogenetic relationships among the Chenopodes utilized sequence analysis based on a combination of chloroplast genes

(*rbc*L, *rbc*S, *mat*K) and nuclear genes (*UNUSUAL FLORAL ORGANS* (*UFO*), *AG, AP3,* and *PI*) from multiple members of the subfamily (Naeger & Golenberg, 2016). This analysis produced a phylogenetic tree with *Chenopodium album*, a hermaphrodite, as the most basal member from which two sister groups are derived. One group is monophyletic and contains the dioecious spinach. The sister group is monophyletic and termed the Blitum group which contain *Blitum bonus-henricus* a protogynecious hermaphrodite (pistil and stigma exposed initally, followed by the stamens) and *Blitum nuttallianum* and *Blitum virgatum*, both of which are gynomonoecious (producing hermaphroditic and pistillate flowers). These observations indicate that dioecious *S. oleracea* likely evolved from a hermaphroditic ancestor.

Dioecious species can be grouped into two classes based on the developmental mechanism that results in a unisexual flower (Mitchell & Diggle, 2005). Type I unisexual flowers begin developing a perfect flower and become unisexual by terminating the development of the gynoecium (female reproductive organs) or the androecium (male reproductive organs), the developmental stage in which organ abortion occurs is species specific. The flower of a Type II species is fated to become pistallate or stamenate during the transition to flowering or soon thereafter. The flower primordia of a Type II species only initiates one type of sex organ and therefore does not require controlled organ abortion however, they are sometimes confused with very early aborting Type I species. *Spinacia oleracea* L. is an example of a Type II dioecious species, thus unisexual flowers are initiated from primordia and can be identified visually (with the help of SEM) based on the development of the first whorl. Female *S. oleracea* flower primordia develop two large sepals from the first whorl of organs that overgrow and protect the developing ovules very quickly.

Male *S. oleracea* initiate four sepals that surround but do not overgrow the stamen primordia until later in flower development. Unlike many angiosperms spinach, regardless of sex, do not produce petals at any point in flower development. As the female flower matures the two sepals continue to cover the stigma while the lower third of the sepals may fuse together. Tendril like stigma protrude past the sepals to catch pollen. By the time the male flower is observable with the unaided eye, the four sepals have overgrown the stamen that develop in an opposite phyllotaxic pattern. As the stamen mature, they force open the sepals and rapidly elongate the filament just before releasing pollen (Sather, D Noah *et al.*, 2005).

At the molecular level, sex can be determined based on expression of B-class genes in the inflorescences which is male specific. B-class gene expression is observed very early and throughout the flower primordia before any organs can be distinguished. As development progresses, the expression pattern of *SpAP3* and *SpPI* become restricted to the stamen primordia (Pfent, Catherine *et al.*, 2005). As the anther locules develops expression of *SpAP3* and *SpPI* is observed in the tapetum and microsporangia (Pfent, Catherine *et al.*, 2005). No expression of either B-class gene was observed via *in situ* hybridization in female flowers at any time during development. However, weak signal was observed in female inflorescences via norther blot for *SpAP3* but no expression was observed for *SpPI (Pfent, Catherine et al., 2005)*. The expression pattern of the C-class *SpAG* in male flowers is observed in the floral primordia which is then restricted to the stamen at later stages (Sather, D Noah *et al.*, 2005). As the anther locules develop *SpAG* expression is observed in the mircosporangium but not the tapetum (Sather, D Noah *et al.*, 2005). During female flower development *SpAG* was observed in the flower primordia and then restricted to the carpel and girdle tissues. As the organ primordia develop, the girdle tissue surrounds the ovule, eventually forming the ovary

wall and pistil from which the stigma will emerge. As development continues *SpAG* expression is restricted to integument within the ovule and is not seen in the ovary wall or girdle tissue (Sather, D Noah *et al.*, 2005). Although expression of B-class genes is male specific, sequence analysis revealed no allelic differences between male and female copies of both *SpAP3* and *SpPI* (Sather *et al.*, 2010). Similarly, no allelic differences were observed between male and female copies of *SpAG* (Naeger & Golenberg, 2016).

Altering the expression of spinach B- and C-class genes through viral induced gene silencing (VIGS) illuminated the roles of each class of organ identity gene. Knocking down expression of *SpAG* produced a lack of flower determinacy resulting in an excess of whorls in both male and female spinach, which is consistent with *AG* function observed in *A. thaliana (Sather et al., 2010)*. In both males and females, the extra whorls typically developed into sepals as C-class function is not required to produce sepals. In females no carpels were observed to develop which is consistent with the ABCE model and in males flattened structures did develop but no pollen or anthers were produced (Sather *et al.*, 2010). Knocking down B-class expression in females had no phenotypic effect. However, in males this did not result in the generation of sterile flowers but caused homeotic and gender transformations. Without B-class expression males with homeotic transformations were observed with one or more stamen converted into carpels. Additionally, some males developed wild-type stamen but produced a carpel in the central whorl (Sather *et al.*, 2010; West & Golenberg, 2018). A few males were observed to produce the characteristic four sepals, a carpel in central whorl, but no stamens. Wild-type female flowers were also frequently observed in knock-down treated male plants (Sather *et al.*, 2010; West & Golenberg, 2018). These results indicate that spinach B-class genes have a novel function

required to suppress the development of carpel tissue in addition to their canonical functions. These observations showcase the importance of properly controlling the expression of B-class genes in spinach and that altering this expression can alter the sex of the individual.

**Genetics of Sex Determination in Spinach**

Spinach is typically dioecious with an even ratio of female to male individuals however, monoecious individuals do naturally occur although with much variety in staminate to pistillate ratio both within and between monoecious individuals, in addition perfect flowers are observed although very rarely (Rosa, 1925). The dioecious character was believed to be controlled by a single locus on chromosome 1 wherein females are homozygous (XX) and males heterozygous (XY) (Janick & Stevenson, 1955). The Y chromosome was observed to be active where a single copy is sufficient for male determination regardless of the number of X chromosomes that accompany the Y (Mahoney *et al.*, 1959). This single locus method of gender determination is adequate for the explanation of dioecy in spinach but not for the natural occurrence of monoecy and quite insufficient for the sexual plasticity observed as a result of environmental factors (Thompson, 1955). Multiple hypothesis were proposed to explain monoecy (Sugimoto, 1947; Bemis & Wilson, 1953b) however, Janick and Stevenson (1955) showed experimentally that monoecy is controlled by a partially dominant allele ($X^m$) of the sex determining XY factor. The Y allele is dominant to X and $X^m$ causing the development of staminate flowers. The partially dominant $X^m$ allele when homozygous produces monoecious individuals that have a male skewed flower ratio. While in the heterozygous arrangement (X $X^m$) a monoecious individual with a female skewed flower ratio is observed. However, when testing the three allele hypothesis in another variety of

spinach Iizuka and Janick observed the P.I. 169671 variety would produce completely staminate plants when homozygous for the $X^m$ allele (Iizuka & Janick, 1962). To account for these data the three allele, one locus hypothesis must be altered to include a variety of $X^m$ alleles that differ in the ability to induce maleness. Alternatively, this observation could also be explained by a two locus system in which the X/Y function is modified by an independent gene (M) that influences the monoecious character.

Recent efforts using multiple marker-based analysis have been focused on determining which of the two hypotheses are correct. Using a combination of microsatellite, amplification fragment length polymorphism (AFLP) and sequence-characterized amplified region (SCAR) markers a map of chromosome 1 was constructed (Khattak, JZ *et al.*, 2006; Onodera *et al.*, 2008; Onodera *et al.*, 2011; Yamamoto *et al.*, 2014). Utilizing the newly created markers and through a series of careful breeding strategies the data suggested that indeed the M locus was independent but linked to the X/Y locus (Yamamoto *et al.*, 2014). The recombination frequency between M and the X/Y locus was observed to be approximately 12% and M was located in a 7.1cM region between SP_0008 and SP_0022 SCAR markers (Yamamoto *et al.*, 2014). The Y allele was observed to co-segregate with markers T11A and V20A in 677 (Akamatsu *et al.*, 1998) and 415 (Yamamoto *et al.*, 2014) plants, suggesting recombination is severely repressed at that locus. To characterize the male determining region a BAC library was created and then parsed using T11A, V20A, and three additional non-recombining markers in coupling phase with the Y allele (Kudoh *et al.*, 2018). None of the BAC clones in the library were positive for more than one of the markers thus five BAC contigs were assembled, one contig per marker. The size of the contigs ranged from 106kb to 180kb and covered a total length of 692kb (Kudoh *et al.*, 2018). Sequence analysis of the

BAC clones revealed only ~4% of the BAC library was homologus to none or one other segment of the spinach draft genome indicating a high level of repetitive sequence and low gene content. Indeed, gene prediction analysis produced only 45 potential open reading frames, 14 of these had no homology to any sequence in the NBCI non-redundant protein database and the rest were either uncharacterized, hypothetical, or retroelement related (Kudoh *et al.*, 2018). The identity of the X/Y sex determining gene and monoecious gene will likely remain unknown for some time given the difficulty of sequencing highly repetitive genomic areas, increasing marker saturation may help in this endeavor.

While we eagerly await identification and characterization of genes associated with the aforementioned markers this does not preclude us from investigating how unisexual flower are produced in spinach. This has been the focus of my doctoral thesis and the opening chapter sheds light on the regulatory genes involved in unisexual development and hypothesizes a mechanism to explain the selective activation of spinach B-class genes. To better understand the suite of genes deferentially expressed between the gender morphs transcriptome analysis was performed and will be reported here. Additionally, the last chapter reports an early unfinished project that sought to characterize any physical interactions between the transcription regulators proposed to be involved in unisexual flower development.

# CHAPTER 2: GENDER SPECIFIC EXPRESSION OF *GIBBERELLIC ACID INSENSITIVE* IS CRITICAL FOR UNISEXUAL ORGAN INITIATION IN DIOECIOUS *SPINACIA OLERACEA* L.

This chapter has been published

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## ABSTRACT

- While unisexual flowers have evolved repeatedly throughout angiosperm families, the actual identification of sex determining genes has been elusive, and their regulation within populations remains largely undefined. Here, we test the mechanism of the feminization pathway in cultivated spinach, and how this pathway may regulate alternative sexual development.
- We tested the effect of GA on sex determination through exogenous applications of GA and inhibitors of GA synthesis and proteasome activity. GA concentrations in multiple tissues were estimated by ELISA analysis. Gene function and pathway analysis were tested through VIGS mediated gene silencing. Relative gene expression levels were estimated by qRT-PCR.
- Inhibition of GA production and proteasome activity feminizes male flowers. However, there is no difference in GA content in tissues between males and females. We characterized a single DELLA transcription factor gene (*SpGAI*) and observed inflorescence expression in females two-fold higher than in males. Reduction of

*SpGAI* expression in females to male levels phenocopies exogenous GA application with respect to flower development.

- These results implicate SpGAI as the feminizing factor in spinach, and suggests the feminizing pathway is epistatic to the masculinizing pathway. We present a unified model for alternative sexual development and discuss the implications to established theory.

## INTRODUCTION

The concept of sex determination in angiosperms is complex. The hermaphroditic flower is generally considered to be the ancestral state of all extant flowering plants. As such, genes that regulate the developmental pathways that lead to sporophytic sexual organs, the stamens and pistils, to alternative gametophytes, the pollen grains and the megagametophyte, and to the actual gametes themselves, the sperm and eggs, are present and shared among species. Sex determination that leads to the production of unisexual flowers, whether in monoecious or dioecious species, must therefore be investigated in terms of the alternative expression of genetic modules that control the development of these structures rather than in terms of the genes themselves alone.

Given that the presence of both sexual organs and functions in a single flower is ancestral, the evolution of unisexuality is best thought of as the accumulation of mutations that ultimately suppress the production of alternative sexual gametes from a single meristem. Specifically, for the evolution of dioecy from hermaphroditism, at least three mutations are required, one to suppress gynoecium development, another to suppress androecium development, and lastly a mutation to suppress recombination between the

previous two (Charlesworth & Charlesworth, 1978). Suppression of recombination between the two sterilizing mutations is necessary to avoid producing sterile offspring. This is commonly thought to be caused by a chromosomal inversion, however, other modifiers that reduce recombination between such loci may also occur. Chromosomal regions with suppressed recombination that also segregate with sex would therefore be primary candidates for the location of sex determining mutations. The suppression of recombination and the reduction of effective population size are theoretically thought to lead to Y (or W) chromosome degradation, and, ultimately, to heteromorphic chromosomes (Charlesworth & Charlesworth, 2000). Identification of these regions is somewhat straightforward in unisexual species with heteromorphic sex chromosomes such as *Silene latifolia* (Westergaard, 1958) that use an XY system, but not simple in species with proposed homomorphic sex chromosomes such as *Spinacia oleracea* (Bemis & Wilson, 1953a; Khattak, JZ *et al.*, 2006). Regardless of sex chromosome morphology, identification of genes predicted to be within non-recombining regions is difficult as these regions tend to accumulate repetitive DNA elements. *Silene* is the best studied of genera with heteromorphic chromosomes and although its Y chromosome harbors numerous intact genes, some with X-linked counterparts, few of these genes are observed to have floral development function (Matsunaga, 2006). Among those genes found on the Y chromosome and known to be involved in flower development are an *APETALLA 3* orhtolog (Matsunaga et al., 2003; Nishiyama et al., 2010), two *SEPALLATA* orthologs (Matsunaga et al., 2004), and two *WUSCHEL* orthologs (Kazama et al., 2012). However, it is not clear if any of these genes function in a sexually deterministic fashion.

In monoecious species unisexuality is not expected to culminate in the development of sex chromosomes and need not follow the series of mutations predicted by Charlesworth and Charlesworth (1978). Although sex determining genes must exist in these species, they cannot be regulated through segregation of alleles as the floral meristems within a plant will all have the same genotype regardless of the gender of the flower produced. Alternative regulation of these sex determining genes would allow for the development of alternate sexual organs within a single individual plant. In monoecious species in which sex-determination has been studied genetically, genes that control the alternative development of flower gender tend to trigger regulatory pathways leading to feminizing or masculinizing development (Golenberg & West, 2013). In most cases, these are not unique sex-specific genes, but rather genes associated with common plant hormone systems. Work in the common melon, *Cucumis melo*, has identified two loci that contribute to sexual determination. Melons can produce monoecious (AAGG), andromonoecious (aaGG), gynoecious (AAgg), and hermaphroditic (aagg) individuals (Poole & Grimball, 1939; Kenigsbuch & Cohen, 1990). The A locus was identified as *CmACS-7* a member of the ethylene biosynthesis pathway (Boualem et al., 2008), the G locus was identified as *CmWIP1* (Martin et al., 2009) a C2H2 zinc-finger transcription factor whose function in Arabidopsis has been shown to be involved in development of female structures in the carpel (Sagasser et al., 2002; Crawford et al., 2007). Recently an upstream gene *CmACS11* has been identified that epistaticly controls the alternative expression of *CmWIP1* and *CmACS7* (Boualem et al., 2015).. Identification of the A locus as a member of a hormone biosynthesis pathway fits well with previous observations of melon sexual determination being influenced by the application of ethylene (Byers et al., 1972). The influence of hormones on sex determination

is not limited to *C. melo* and has been observed in other Curcubits (McMurray & Miller, 1968) *Mercurialis annua* (Durand & Durand, 1991) and in maize (Bensen et al., 1995). However, the hormone correlated to sex determination differs between species: ethylene in Curcubits (Trebitsh et al., 1997; Mibus & Tatlioglu, 2004); auxin and kinetin/cytokinin in *M. annua* (Dauphin-Guerin et al., 1980; Hamdi et al., 1987); and gibberellic acid/jasmonic acid in maize (Hansen *et al.*, 1976a; Acosta *et al.*, 2009). These data have improved our understanding of the genes and hormones able to influence sexual determination, but little progress has been made in understanding how these determining factors regulate downstream pathways to cause differential sexual development.

Spinach (*Spinacia oleracea*) is a dioecious plant that has been domesticated and valued for its highly nutritious leaves. The agricultural importance, hardy and reliable growth, simplified flower structures, diploid genome with low chromosome number (2n=12), and rapid maturity make spinach an excellent organism to study the genetic mechanisms controlling sexual development in dioecious species. Spinach plants develop unisexual flowers without any intermediate hermaphroditic stage indicating that sexual determination occurs early in floral primordia development or during the transition to flowering (Pfent, C. *et al.*, 2005). Female spinach flowers develop two sepals that surround the ovary and partially fuse along their edge. Multiple stigma lobes protrude from between the sepals. Male flowers produce four sepals that do not fuse, four stamens and no central organ. Neither male nor female flowers develop petals (Sherry et al., 1993; Sather et al., 2005). Early studies have indicated the presence of a sex-determining locus on the longest chromosome in spinach (Janick & Stevenson, 1955; Janick *et al.*, 1959; Mahoney *et al.*, 1959), however, there is no consistent evidence of heteromorphic sex chromosomes (Ramanna,

1976). There are reports of heteromorphic chromosomes in some isolated accessions of the congener *Spinacia tetrandra*, but these are not found in all accessions of that species or in *Spinacia oleracea* or *Spinacia turkestanica* (Fujito *et al.*, 2015) . Similarly, sex-specific markers have been reported (Khattak, J *et al.*, 2006; Lan *et al.*, 2006; Onodera *et al.*, 2011) to be linked to a single sex determining gene on chromosome 1, however these markers are identified in restricted accessions and do not always remain linked with sex in different accessions (Fujito *et al.*, 2015). The most recent draft of the spinach genome (Xu *et al.*, 2017) has not identified sex-specific chromosomal regions. Therefore, while a heterozygous/homozygous sex determination system is accepted, the question of whether such a gene or gene cluster is embedded in non-recombining chromosomal region remains unresolved.

Previously we have observed that the spinach B class genes *SpPISTILLATA* (*SpPI*) and *SpAPETALLA3* (*SpAP3*) begin to be expressed in early male flower primordia prior to the initiation of organ primordia, but are not expressed at any time in female flowers (Pfent, C. *et al.*, 2005). Work using a virus induced gene silencing (VIGS) vector pWSRi (Sather & Golenberg, 2009) indicated that silencing of B class gene expression in male spinach plants resulted in mosaic individuals with homeotic transformations of stamens into carpels and the formation of gynoecia in the normally absent fourth whorl (Sather et al., 2010). This indicates that the B class genes are not only responsible for the canonical B class role of stamen determination but also possess a novel function required for the suppression of female fourth whorl organs. Therefore, B-class genes are acting as the masculinizing genes in spinach. The suppression of B-class expression during early flower development leads to female commitment. Thus, the spinach feminizing factor prevents B class expression and is

therefore epistatic to the male determining genes. The identity of such feminizing factors remains unclear.

Previous work has shown that the phytohormone gibberellic acid (GA) can influence spinach sexual development. The application of GA to media of hydroponically grown spinach was enough to masculinize 78% of the treatment group (Chailakhyan, MK & Khryanin, VN, 1978). We can infer that increased GA concentration is able to initiate expression of B class genes. Similar to other phytohormones, GA perception initiates the degradation of repressive transcription factors that then allows the activation of hormone response genes (Spartz, A. K. & Gray, W. M., 2008). The DELLA family of transcription factors has been observed to function as negative regulators of GA signaling (Peng et al., 1997; Silverstone et al., 1998). GA is perceived by a receptor GIBBERELLIN INSENSITIVE DWARF1 (GID1) which is observed to interact with DELLA family proteins in a GA dependent manner (Ueguchi-Tanaka et al., 2005). This interaction causes a conformational change in the GID1 protein allowing it to bind the F-box component of a SCF E3 ubiquitin ligase (Murase et al., 2008), which leads to GAI degradation mediated by the 26S proteasome (Fu et al., 2002). The floral F box protein UFO is required for polyubitination and is required for activation of B class genes (Ng & Yanofsky, 2001; Laufs *et al.*, 2003; Ni *et al.*, 2004; Hepworth *et al.*, 2006)(Ng & Yanofsky, 2001; Laufs *et al.*, 2003; Ni *et al.*, 2004; Hepworth *et al.*, 2006). There appears to be a single spinach homolog of UFO, and it is expressed in both male and female flowers (J. A. Naeger and E. M. Golenberg, unpublished data).

In this study, we investigate the role of GA and the DELLA transcriptional regulators in sex determination of dioecious spinach. The exogenous application of GA and paclobutrazol (PAC), a chemical inhibitor of GA biosynthesis confirmed previous results showing that GA

masculinizes spinach but also revealed the lack of GA feminized spinach. An ELISA assay was used to assess GA concentration in various tissues, however, no difference in GA content between males and females were observed. Using a VIGS based delivery system we silenced the spinach homolog *GIBBERELLIC ACID INSENSITIVE* (*GAI*) a DELLA family member known to be responsive to GA and show that *SpGAI* expression is required for female determination. Silencing of spinach B class gene expression in males had no effect on *SpGAI* expression, indicating that SpGAI acts upstream of the B class genes. However, *SpGAI* is differentially expressed in a gender-specific fashion. Based on these data and incorporating observations from previous work we present a molecular model for sexual determination of dioecious spinach dependent on *SpGAI* expression.

## METHODS

### Plant Material

All plants used in this study are *Spinacia oleracea* cv.America (Ferry-Morse Seed Company). Plants were grown from seed in commercial potting soil and watered every two to three days as needed.

### GA, PAC, and MG132 Application

Spinach plants were grown under flowering conditions (16hrs light, 8hrs dark) at 20ºC until reaching the two-to-four leaf stage at which time approximately 1-2mL of either 50µM GA3 or 50µM paclobutrazol solution were sprayed onto spinach plant leaves and stems. The control group was sprayed with water only. The solutions were applied in separate fume hoods to eliminate the possibility of accidental cross-treatment. Once applied the treated flats remained in the fume hood until the solution present on the plant had dried before being returned to separate growth chambers. Flats of spinach were treated in this manner every

three to four days for two weeks. MG132 was applied directly to early emerging inflorescence meristems. The treatment group received 50µL of a 50µM MG132 and 0.02% Tween-20 solution. The control group received 50µL application of 0.02% Tween-20. Resulting morphology was photographed using on an Olympus SZX 16 Dissecting Microscope.

**GA quantification**

Approximately 10mg of tissue from juvenile leaves, mature leaves, inflorescence meristem, and mature flowers were harvested from eight male and female individuals. 500uL 1xPBS added to dissected tissue and ground with mortar and pestle, then more thoroughly homogenized by sonication. Homogenate was centrifuged to pellet cellular debris and the supernatant transferred to a clean tube for use in ELISA assay. GA content of tissue samples was analyzed following the protocol accompanying Plant Gibberellic Acid, GA, ELIAS kit from MyBioScource (Catalog# MBS9310617). Two-way ANOVA calculations were used to determine statistical significance.

**SpGAI isolation**

DELLA protein sequences were downloaded from GenBank for A. thaliana RGA1 (CAA72177), RGA2 (CAA72178), GAI (NP_172945), Glycine max GAi1 (ABO61516), Vitis vinifera predicted GAI1 (XP_002284648), and Malus X domestica GAI1 (ACL68360) and aligned to detect conserved amino acid sequences downstream of the DELLA conserved regions. Degenerate primers were designed (Supplemental Table 1) and the resultant PCR product was cloned and sequenced. The remainder of the sequence was determined by 3'RACE and 5' Splinkerette PCR (Devon et al., 1995). Additional sequences were downloaded for comparisons from *Cucurbita maxima* (Q6EI05), *Populus trichocarpel* (XP_002305198),

*Gossypium barbadense* (ABG26370), *Sinningia speciosa* (ACM47244), *Theobroma cacao* (XP_007045197), *Beta vulgaris* (XP_010681882), and *Ricinus communis* (EEF34604). We aligned the predicted translated amino acid using the Geneious alignment tool with a BLOSUM62 cost matrix (Gap penalty 12, Gap extension penalty 3) and estimated a phylogenetic tree using a Neighbor-joining Geneious application (Kearse et al., 2012).

**VIGS based knockdown of GAI and PI expression**

The gene silencing vector pWSRi derived from the Beet Curley Top Virus was used for gene silencing (Golenberg et al., 2009). In general, a 200 bp fragment is cloned into the vector where the sequence will be transcribed in planta in both directions. Sequences for the pWSRi:SpAP3 and pWSRi:SpPI have been previously published (Pfent, C. *et al.*, 2005). To construct the pWSRi:SpGAI vector, we subcloned a 400bp fragment of SpGAI from the variable region 3' of the DELLA sequence (but including the VHYNP encoding sequence) into our silencing vector pWSRi. Individual *Spinacia oleracea* plants were biolistically bombarded with pWSRi: Empty Vector (negative control), pWSRi: SpGAI, or pWSRi: SpPI coated tungsten bullets once they matured to the two-to-four leaf stage. The BioRad Helios Gene Gun was used for biolistic bombardment and plants where shot once per plant at ~80psi. Bullets were created following the manufactures instructions while combining 25mg tungsten with ~50µg pWSRi: GOI or pWSRi: Empty Vector plasmid DNA in ~25in. of tubing which results in delivering ~1µg of pWSRi: GOI or pWSRi: Empty Vector per target. After bombardment, the plants were placed under a plastic wrap tent for 24hrs to keep local humidity elevated. The treated spinach was grown under flowering conditions (16hrs light, 8hrs dark) at 20ºC in Conviron growth chambers. Treated plants were classified as female or male based on the predominant flower type and inflorescence architecture. Specifically,

female flowers are noted by the presence of two sepals and a central carpel, whereas male flowers are distinguished by the presence of four sepals and stamen. Female inflorescences in cv America have prominent leaves on the axis and flowers in the leaf axils. Male inflorescences have much reduced leaves on the inflorescence. Resulting phenotypes were photographed on an Olympus SZX 16 Dissecting Microscope approximately 4 weeks post bombardment.

**qPCR analysis of VIGS based silencing**

Floral inflorescence RNA samples from multiple male and female spinach individuals in the control group, and multiple mixed flower individuals from the treatment group were extracted and purified using the RNeasy plant RNA extraction kit from Qiagen. cDNA was created following the ClonTech RNA to cDNA EcoDry Premix kit protocol using 3µg of sample RNA as template. qPCR was performed on Agilent Technologies Mx3000P machine using iTaq Universal SYBR Green Supermix buffer from BioRad. Primer information can be found in Supplemental Table 1. Amplification conditions were as follows: Denaturation at 96ºC for 3min, then 40 cycles composed of 30sec denaturing at 96ºC, annealing at 55ºC for 30sec, and extension at 72ºC for 30sec. Melting point profiles were examined to confirm that single PCR products were produced. Expression values were determined with the ΔΔCt method using UBQ5 as the internal reference gene (Gutierrez et al., 2008) when calculating GAI expression the female sample was set as the calibrator sample. When calculating PI expression the male sample was set as the calibrator sample. One-way ANOVA calculations and Tukey test were performed on the data to determine statistical significance.

## RESULTS

**GA Influences Sex Development in Spinach**

Previous studies that applied exogenous GA either through hydroponic or direct application demonstrated the GA has a masculinizing effect in spinach (Chailakhyan & Khryanin, 1978; Chailakhyan & Khryanin, 1979). We expanded upon these studies to include paclobutrazol (PAC), a chemical inhibitor of GA synthesis and utilized a topical spray of PAC and GA for application. After 3 weeks, the GA treated individuals were taller and had larger leaves compared to control plants. As predicted the PAC treated individuals exhibited a phenotype opposite to exogenous GA application growing shorter than untreated plants with smaller, dark green leaves. Additionally, the PAC treatment group flowered three weeks later than untreated and GA treated groups, which flowered at approximately the same time.

The GA treated females produced inflorescences that contained a range of floral morphologies including wild-type female (Figure 1A), female with ectopic stamen (Figure 1B), and wild-type male flowers. The degree to which stamen develop and carpels are suppressed (masculinization) can vary from mild to severe. Mild masculinization exhibits a single stamen that develops within two sepals alongside one pistil (Figure 1B). Moderate masculinization displays a flower with two sepals where the pistil has been replaced with one or more stamens (Supplemental Figure 1A). Severely modified females have four sepals, four stamens, and a single pistil (Supplemental Figure 1B). We did not observe any unusual floral phenotypes in male plants treated with GA. These data are consistent with previous observations and support the notion that GA promotes male development in spinach.

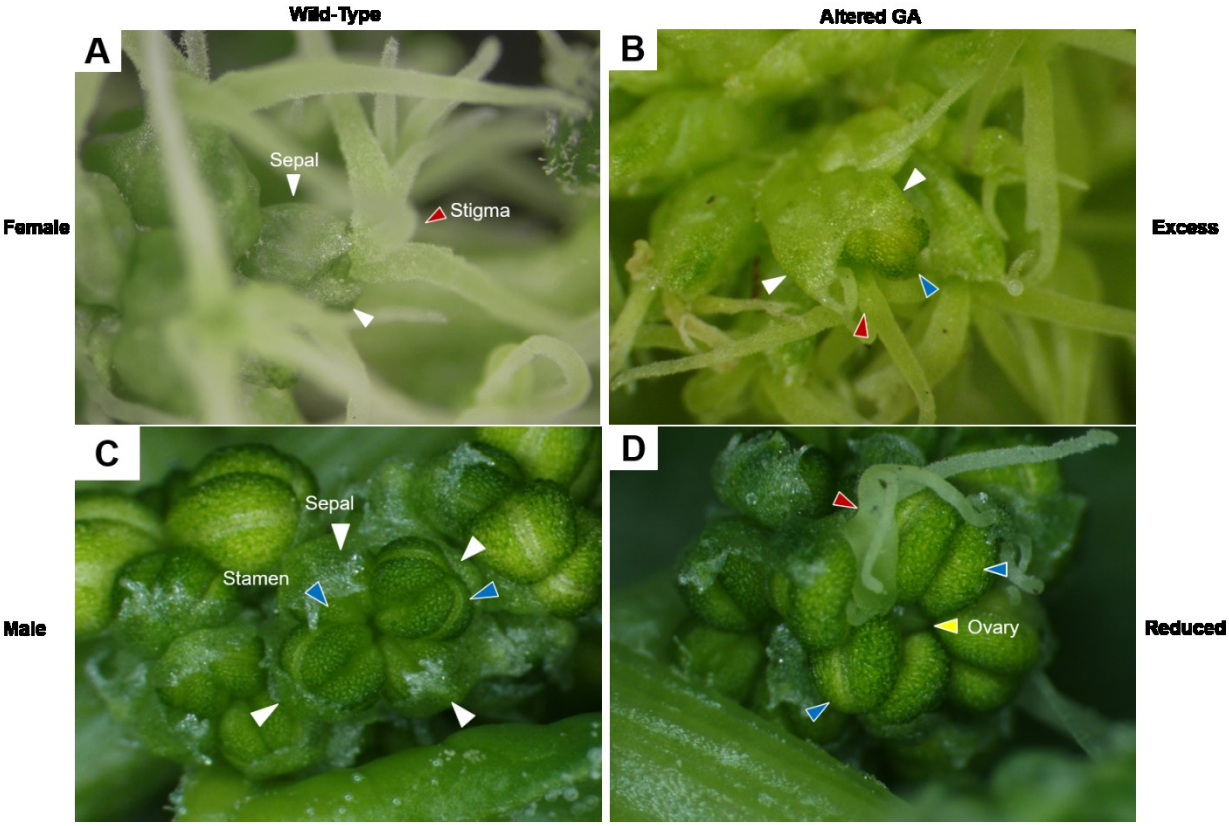


***Figure 2.1*** *– Exogenous application of GA3 and PAC onto Spinacia oleracea.* ***(A)*** *and* ***(C)*** *are wild-type female and male flowers respectively.* ***(B)*** *Masculinized female flower resulting from GA3 application with two sepals, one stamen, and one pistil.* ***(D)*** *Feminized male flowers resulting from PAC application with a single stigma and central ovary. White arrow = sepals, blue arrow = stamen, red arrow = stigma, yellow arrow = ovary.*

In contrast to GA treatment, PAC treatment reduced stamen development and induced pistil development in male spinach plants. PAC application produced a range of phenotypes. Compared to wild-type male flowers (Figure 1C) moderately affected males would often display homeotic conversion of one or more stamens into female organs including stigma or complete pistils, sometimes accompanied by development of a gynoecium in the fourth whorl (Figure 1D, Supplemental Figure 2A). Severely affected male flowers retained four sepals but developed a wild-type pistil and no stamen (Supplemental Figure 2B). Notably,

some male flowers yielded an incomplete homeotic conversion where stigma would develop from stamen tissue (Supplemental Figure 2C). The female plants were not observed to have any floral modifications following PAC treatment. Taken together these results indicate that an excess of GA causes the production of male organs in females while reducing GA content promoted female organ development in males.

**Proteasome Function Required for Male Development**

DELLA family transcription factors are known to repress GA response genes and GA is observed to facilitate the degradation of DELLA repressors thus allowing the expression of GA response genes (Peng et al., 1997; Silverstone et al., 1998). This GA initiated DELLA degradation is achieved through processing by the 26S proteasome (Fu et al., 2002; Dill et al., 2004). To determine if the observed influence of GA on sexual development requires the 26S proteasome, and thus potentially DELLA transcriptional repressors, we exogenously applied MG132, a chemical inhibitor of the 26S proteasome (Rock et al., 1994). Upon flowering, we observed development of female organs in male individuals (feminization) while no unusual phenotypes were observed in female plants. Compared to wild-type males (Figure 2A) affected male flowers were observed developing stigma (Figure 2B) and gynoecium (Figure 2D) within otherwise male flowers. This phenotype resembles the feminization phenotype observed with PAC treatment (Figure 2C and 2D). Thus, reduction of GA or inhibition of proteasome mediated protein degradation produces the same developmental response. This similarity suggests that both GA hormone and 26S proteasome function are required for male development indicating that androecium initiation results from the degradation of an inhibitor in response to GA hormone signaling.

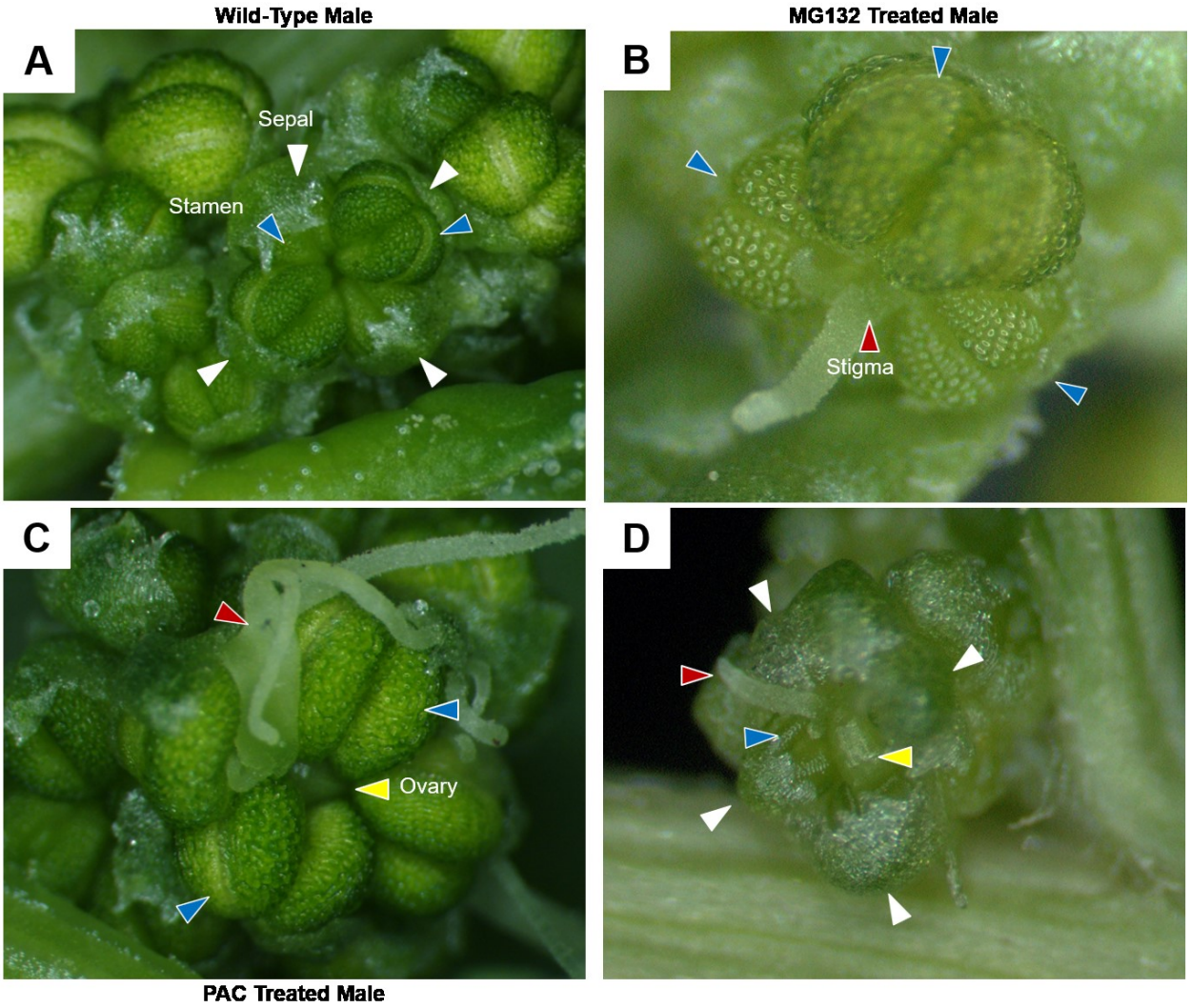


***Figure 2.2*** *– Exogenous application of MG132 onto Spinacia oleracea.* ***(A)*** *Wild type male flower.* ***(B)*** *Feminized male flower resulting from MG132 application with central stigma and* ***(D)*** *another example with an ovary developing in the fourth whorl.* ***(C)*** *Feminized males observed after PAC treatment. White arrow = sepals, blue arrow = stamen, red arrow = stigma, yellow arrow = ovary.*

**GA content in male and female tissues**

In order to investigate if there is a difference in GA content between males and females, we prepared tissue homogenate derived from mature inflorescences, the inflorescence meristem region, juvenile leaves, and mature leaves of eight male and eight female plants. All samples of juvenile leaves were harvested at the second leaf stage, inflorescence

meristem samples were harvested between the sixth and ninth leaf stage. Mature leaves were taken after bolting had begun and flower inflorescences were sampled before dehiscence or fertilization occurred. The GA content of these samples was analyzed via ELISA and a two-way ANOVA (Figure 3). There was no significant interaction between tissue type and gender ($F_{3,47}$= 0.3049 NS), nor significant effect by gender ($F_{1,47}$= 0.0457 NS). GA content did vary significantly by tissue type ($F_{3,47}$= 49.37, $p < 0.001$). The inflorescence apical region was observed to have the highest concentration of GA while juvenile leaves had the least amount of GA. Mature leaves and mature flowers had a similar amount of GA content. This is consistent with reports from other species (Silverstone et al., 1997). These data indicate that GA content does not portend sexual development but perturbation of GA concentration will influence sex in a predictable fashion.

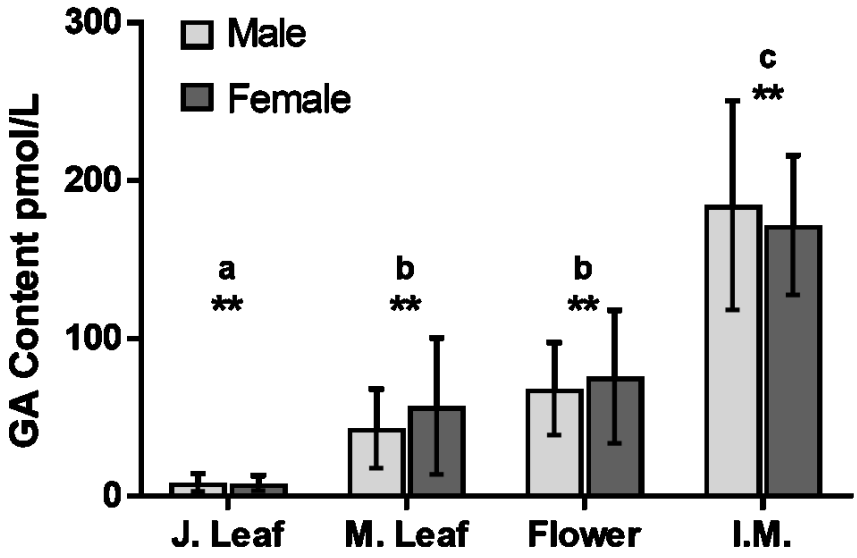


***Figure 2.3*** *– GA content analysis of Spinacia oleracea tissue homogenate. Eight male and female individuals analyzed for GA hormone content. Error bars represent SD (n = 6 independent samples), asterisks indicate p<0.01 from Two-way ANOVA analysis and Tukey test, letters indicate statistically significant groups. J. Leaf = Juvenile Leaf, M. Leaf = Mature Leaf, I.M. = Inflorescence Meristem*

### *SpGAI* Expression is Required for Female Development

Based on these observations, the sex determination in spinach cannot be explained through differential production of GA, but must be responsive to GA signaling. Our present results suggest that the female determining protein is degraded by the 26S proteasome. GA response genes are known to be regulated by DELLA transcriptional repressors that are also understood to be degraded by the 26S proteasome (Sasaki et al., 2003; Dill et al., 2004). Taken together these observations suggest that a spinach DELLA protein is required for female development and must be degraded to allow for male development. *Arabidopsis thaliana* is known to possess five DELLA transcription factors while other species, such as rice, have only one copy (Ikeda et al., 2001; Lee et al., 2002).

To isolate DELLA protein genes from *S. oleracea* cv America, degenerate primers were designed to anneal to conserved regions in Arabidopsis GAI, RGA1, RGA2, and proposed DELLA proteins in other eudicots. A 472 bp sequence was amplified and sequenced. The sequence contained conserved amino acid encoding regions and aligned significantly to GAI-like or GRAS DELLA proteins in other species. The entire coding, 3' UTR, and 5' upstream regions were isolated from cDNA and genomic DNA. The complete sequence was amplified as a single PCR product using primers 5' to the start codon and 3' to the stop codon. There are no introns and no detectable variations among male and female spinach individuals in the regions sequenced. The predicted amino acid sequence was then used in phylogenetic analysis and is sister to a *Beta vulagaris* GAI protein (Supplemental Figure 3). As they became available, we compared our sequence to male and female flower transcriptomes (N. W. West and E. M. Golenberg, unpublished) and to the draft *S.oleracea* cv Viroflay genome 1.01 (Dohm et al., 2014). The PCR isolated sequence was 100% identical to the

transcriptome contig and 99.9% identical to the cv Viroflay genomic sequence. In searching the *Spinacia* data bases, we used a 150bp segment of our sequenced *SpGAI* that included the DELLA motif and flanking regions as a query, we were unable to detect an additional DELLA protein. The sequence has been submitted to GenBank (Accession No. KX026951).

To date, we have only detected one copy of a DELLA family transcription repressor present in the spinach genome. We therefore hypothesize that SpGAI is the DELLA protein required for female development and the main target for GA initiated 26S proteasome degradation in male flower development. In order to test this, we cloned *SpGAI* into our VIGS vector and biolistically bombarded spinach at the two-to-four leaf stage. A separate group of plants were bombarded with pWSRi:EmptyVector to serve as a control group.

The pWSRi:GAI and EmptyVector control groups developed at normal rates, suffering minimal damage from the bombardment. Spinach in the pWSRi:EmptyVector control group developed phenotypically normal female or male flowers (Figure 4A and 4C). Females in the pWSRi:GAI group developed a variety of floral phenotypes displaying a range of male organ development. Moderate phenotypes developed stamen in place of pistils but still produced two sepals (Figure 4B). While more severe phenotypes developed four sepals, a single pistil, and a single stamen (Supplemental Figure 4A) as well as flowers with four sepals, four stamen, and a single central pistil, similar to other perfect flowers in the Chenopodiaceae (Supplemental Figure 4B). Males within pWSRi:GAI group developed wild-type male flowers and appeared unaffected by *SpGAI* silencing.

The observation of male organ development on individuals with reduced *SpGAI* expression suggests that SpGAI is able to suppress B-class gene expression. In order to determine if B-class genes are able to influence *SpGAI* expression we replicated the pWSRi:SpPI gene silencing experiment previously reported (Sather et al., 2010) and sampled affected flowers from all treatment groups to analyze gene expression. Compared to wild-type male flowers (Figure 4C), *SpPI* silencing produced inflorescences with a variety of floral phenotypes including wild-type male, wild-type female, and mixed-gender flowers. Occasionally, this phenotypic range was displayed within a single inflorescence (Figure 4D) in which we observe a wild-type male unaffected by knockdown treatment; a hermaphroditic flower with four sepals, four stamen, and single pistil indicating mild *SpPI* knockdown; a moderately affected flower with four sepals, single stamen, and well developed pistil; and severely affected flower that appears as a wild-type female flower.

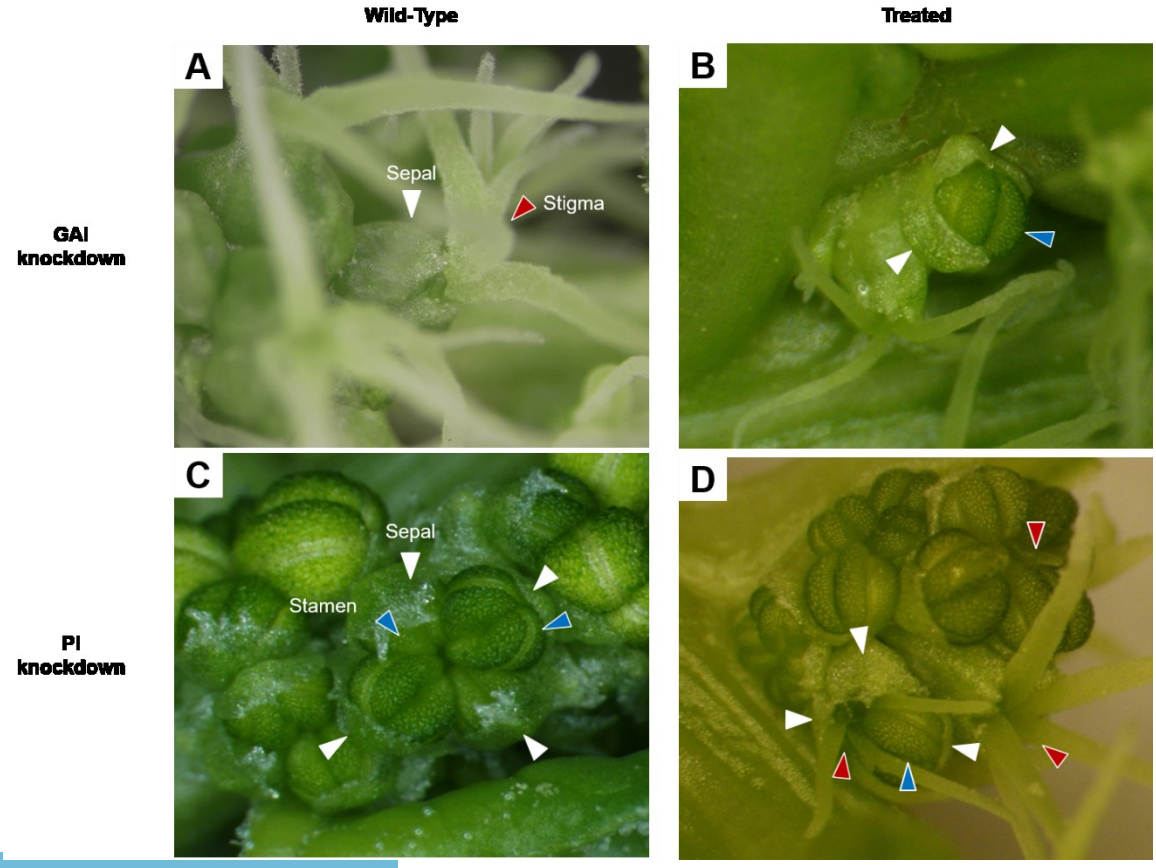

***Figure 2.4*** *– Phenotype resulting from VIGS based knockdown of SpGAI and SpPI in Spinacia oleracea.* ***(A)*** *Wild-type female flower on control treated individual.* ***(B)*** *Masculinized female resulting from SpGAI knockdown with two sepals, one stamen, and no female organs.* ***(C)*** *Wild-type male flowers on control treated individual.* ***(D)*** *Feminized male resulting from SpPI knockdown with four sepals, single stigma and stamen. White arrow = sepals, blue arrow = stamen, red arrow = stigma.*

To asses *SpGAI* expression inflorescence tissue was harvested and RNA was extracted from five individuals of each treatment group. *SpGAI* expression was observed via RT-qPCR and the ΔΔCt method was used to calculate relative expression using female samples as the calibrator. We observe a significant difference in *SpGAI* expression in males compared to female individuals, with males producing roughly half the *SpGAI* mRNA as females (Figure 5A). Females that produced stamens from the *SpGAI* knockdown treatment expressed *SpGAI* at a greatly reduced level when compared to wild-type females although their expression range fell within observed male expression values and were not found to be statistically different from males. Males that developed female organs with pWSRi:PI treatment are observed to have *SpGAI* expression similar to wild-type males. Compared to female levels of *SpGAI* expression, wild-type male, pWSRi:GAI treated females, and pWSRI:PI treated males have statistically significant reduction of *SpGAI* expression ($F=33.58$, $p<0.001$, $df=3$). However, there is no statistically significant difference in *SpGAI* expression among wild-type males, pWSRi:GAI treated females, and pWSRI:PI treated males.

Analysis of *SpPI* expression was conducted by harvesting inflorescence tissue and extracting RNA from three individuals from each treatment group. Characterization of *SpPI* expression was made using qRT-PCR and interpreted using the ΔΔCt method with male expression set to one. *SpPI* expression in wild-type females is non-measurable and follows

previously reported observations (Pfent, C. *et al.*, 2005). Females developing stamens following pWSRi:GAI treatment showed dramatically increased expression of *SpPI* compared to wild-type females, although not quite matching the wild-type male levels of expression (Figure 5B). Males producing female tissue following pWSRi:PI treatment were observed to have significantly reduced *SpPI* expression compared to wild-type males. ANOVA analysis indicates that the observed differences in expression are statistically significant and Tukey testing identified that each sample was significantly different from all others tested ($F=2058$, $p<0.001$, $df=3$). Taken together these results suggest that differential expression of *SpGAI* is necessary and sufficient to initiate *SpPI* expression, which is itself able to simultaneously promote androecium development while suppressing gynoecium development. *SpPI* expression does not appear to influence the expression of *SpGAI* in a significant way, indicating SpPI function is downstream of SpGAI.

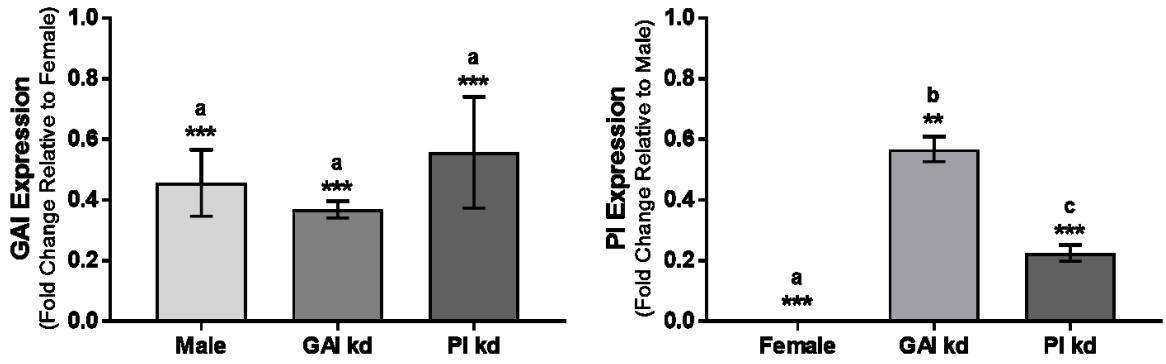


***Figure 2.5*** *– qPCR expression analysis of SpGAI and SpPI in wild-type and treatment groups of Spinacia oleracea.* ***(A)*** *Expression of SpGAI in wild-type males, SpGAI knockdown individuals, and SpPI knockdown individuals. Expression was calculated using the ΔΔCt method setting the female data to 1 as the calibrator sample. Error bars indicate SD (n = 5 independent samples), asterisks represent p<0.001 (***) from one-way ANOVA analysis, letter indicate groups found to be significantly different from Tukey test.* ***(B)*** *Expression of SpPI in wild-type females, SpGAI knockdown individuals, and SpPI*

*knockdown individuals. Expression was calculated using the ΔΔCt method setting the male data to 1 as the calibrator sample. Error bars indicate SD (n = 3 independent samples), asterisks represent p<0.01 (**) and p<0.001 (***) from one-way ANOVA analysis, letter indicate groups found to be significantly different through Tukey test.*

**Sequence Analysis of the 5` Regulatory Region of *SpGAI***

Sex specific sequence variation of this 5` regulatory region could account for the differential expression of *SpGAI* observed in male and female individuals. Regulatory regions that influence transcription in plants are often confined to approximately 1kb upstream of the gene in question (Yu et al., 2016). To determine if the differential expression of *SpGAI* between males and females is due to discrepancies in their cis-regulatory regions we cloned 1280bp fragment from genomic DNA samples of male and female spinach. This 1280bp region included the first 125bp of the *SpGAI* exon and the preceding 1155bp of the 5` regulatory region. Sanger sequencing was performed on the clones and compared to the published genome of *S. oleracea* cv Viroflay 1.01 (Dohm et al., 2014). Analysis revealed 1130 high quality base reads with no sequence differences between male and female samples (Supplemental Figure 5). However, comparison to the published cv Viroflay genome revealed that a 61bp insert is present in the 5` regulatory region in both male and female *S. oleracea GAI* located -621bp from the start codon of *SpGAI* (Figure 6A). Dot plot comparison between *S. oleracea* cv America 5` regulatory region of *SpGAI* and the cv Viroflay genome indicates a region of repetitive sequence elements clustered between -495bp and -737bp from the start codon (Figure 6B, Supplemental Figure 6). Thus, while the region varies

among culitvars of spinach, there is no detectable variation on the nucleotide level that correlates with *SpGAI* expression and gender determination.

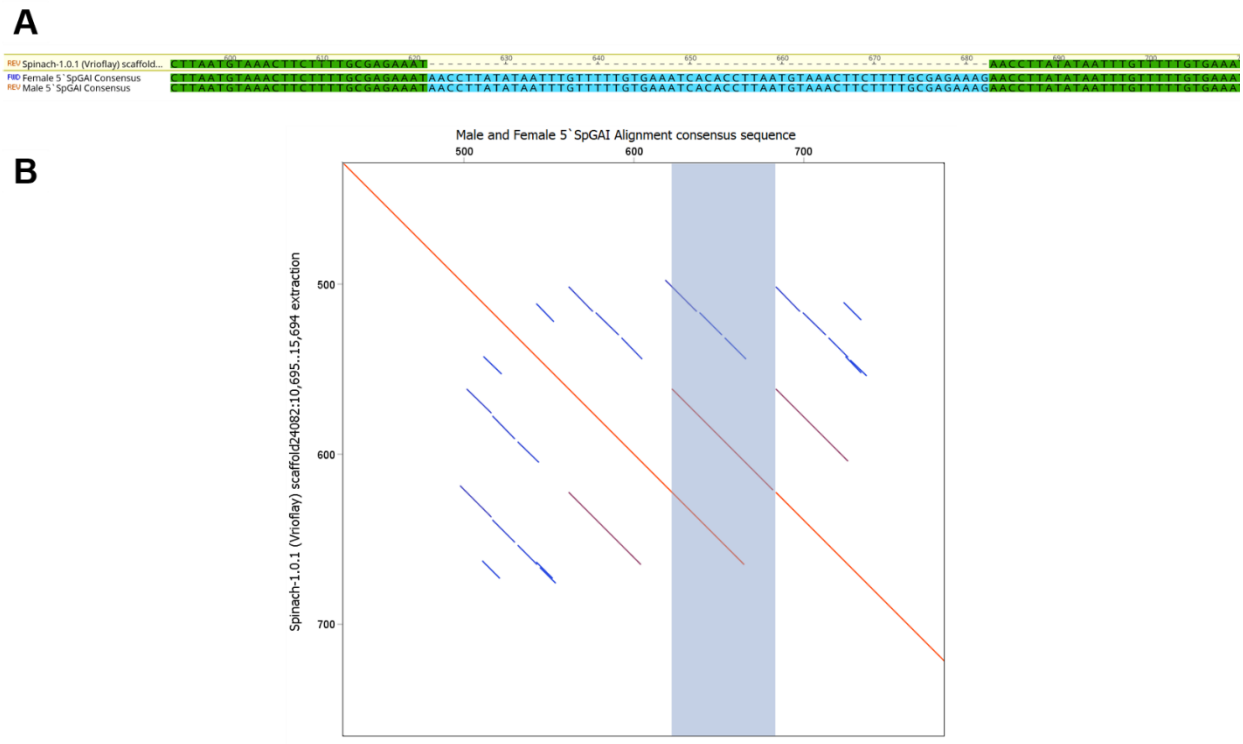


***Figure 2.6*** *– Sequence comparison between male and female SpGAI, and between Spinacia oleracea cv America and cv Viroflay.* ***(A)*** *Sequence alignment of published S. oleracea cv Viroflay, male and female S. oleracea cv America showing a 120bp region with the unique 61bp cv America insertion.* ***(B)*** *Dot plot of SpGAI from S. oleracea cv America and Viroflay indicating numerous repetitive sequences. Blue box highlights the cv America specific 61bp insertion. Blue lines indicate short matches, red indicates a match length over 100bp. Alignment and dot plot created with Geneious software package ver 10.2.2*

## DISCUSSION

We have previously shown that the spinach B-class genes are able to suppress the development of female floral organs and thus is the masculinizing pathway (Sather et al., 2010). Coupled with the current results, we propose a mechanism for sexual determination in spinach that incorporates SpGAI expression and GA content (Figure 7). The expression of SpGAI is required for the development of female organs. SpGAI acts to repress B-class

expression that is necessary for stamen development rather than selectively initiating female development. Consistent with the regulatory hierarchy, we do not observe any significant change in *SpGAI* expression upon B-class silencing suggesting that there is no significant feedback mechanism present and that B-class expression is downstream of SpGAI function. The observation that *SpGAI* is expressed at a higher level in females than in males suggests this transcription repressor may function as a switchpoint determining which sexual development path is initiated. Indeed, upon VIGS mediated knockdown of *SpGAI*, affected females were observed with reduced *SpGAI* expression and also an increased *SpPI* expression. Without any apparent differences in the cis-regulatory region of *SpGAI*, the cause of sex specific differential expression remains elusive but it is likely to involve post-transcriptional regulation and/or epigenetic modifications. It is understood that DELLA transcription factors like SpGAI are targeted for degradation upon GA signaling. Thus, given the same GA signal but differential expression of *SpGAI* the response would differ between males and females. Following the *Arabidopsis* model, expression of a spinach *LEAFY* homologue (*SpFY*) is expected to initiate transition to flowering (Weigel & Nilsson, 1995; Blazquez et al., 1997). In spinach females, due to an excess of SpGAI, which prevents the activation of B-class genes, only the C-class gene *SpAGAMOUS* is initiated resulting in gynoecium development (Sather, D. N. *et al.*, 2005). The low level of SpGAI in males is degraded during transition to flowering allowing *SpLFY* to activate both B-class and C-class genes. C-class expression results in floral determinacy and would typically result in female organ development, however, *SpAP3* and *SpPI* have a novel function that suppresses gynoecium development but does not interfere with C-class determinacy functions resulting in male flowers (Sather, D. N. *et al.*, 2005). Hence the feminizing GA/SpGAI pathway

epistatically suppresses the masculinization pathway resulting in female flowers, while release of the B-class suppression results in gynoecially suppressed male flowers.

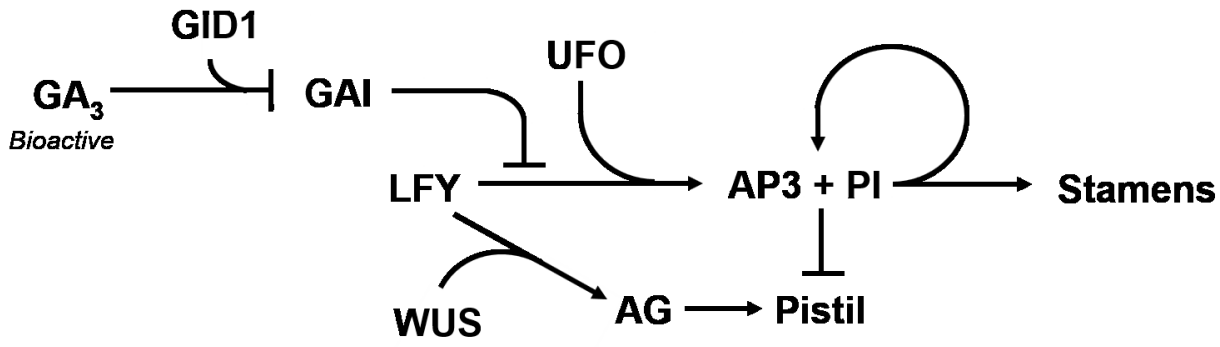


***Figure 2.7*** *– Proposed mechanism for sexual determination in Spinacia oleracea. Arrow heads indicate activation of target genes, "T" heads indicated repression of gene expression or function. Dashed lines indicate indirect interaction. Gibberellic acid (GA) is perceived by its receptor GA INSENSITIVE DWARF1 (GID1), which triggers the degradation of the repressor protein GAI with the aid of the F box protein UNUSUAL FLORAL ORGANS (UFO) and its associated SCF complex. Under permissive conditions of reduced GAI, B class genes are activated. They suppress the formation of the fourth whorl. Under non-permissive, high GAI content, B class genes are suppressed, and only the pistil develops under the effects of the floral C class identity gene AGAMOUS. AGAMOUS also acts to terminate the meristem maintenance gene WUSCHEL (Lohmann et al., 2001).*

The evolution of unisexual flowers from an ancestral hermaphroditic state has evolved independently numerous times. Due to this independence, there is no expectation for the development of a shared method for sexual segregation. Indeed, there is much diversity in the method of segregation. Yet the emerging spinach model fits well with the multiple examples of hormone mediated sex-determination. In melon three genes have been identified to control sexual determination *CmACS11, CmWIP1* and *CmACS7* (Boualem *et al.*, 2008; Martin *et al.*, 2009; Boualem *et al.*, 2015). *CmACS11* is an upstream gene that

epistatically represses *CmWIP1* expression. *CMWIP1* simultaneously suppresses gynoecium formation and the expression of *CmACS7*, while *CmACS7* expression*CmACS7*expression results in the suppression of stamen formation. Thus, ultimately, expression of *CmACS11* determines the development of either male or female flowers (Boualem *et al.*, 2008; Boualem *et al.*, 2015).. CmACS7 is known to function in the ethylene biosynthesis pathway and is required to suppress stamen development in female melon (Boualem et al., 2008). This corresponds well with earlier observations of feminization upon exogenous ethylene application (Byers et al., 1972). In a similar manner, dwarf mutations in maize have been identified as a part of the GA biosynthetic pathway (Hedden & Phinney, 1979; Hedden & Graebe, 1985). Phenotypically the dwarf mutations produce short andromonoecious individuals. The presence of hermaphroditic flowers suggests that these genes are required for arresting male organ development in female floral primordia. In maize the *tassel seed* (*ts*) mutations were observed to feminize the male flowers through the failure to abort pistils (Emerson, 1920). The tassel seed mutations have been identified as parts of the jasmonic acid biosynthetic pathway (Calderon-Urrea & Dellaporta, 1999; Acosta et al., 2009). Exogenous application of the proper hormone reverses the sexual defects observed in *tassel seed* and *dwarf* mutants suggesting that hormones play a vital role in sexual determination (Phinney, 1956; Acosta et al., 2009). Crosstalk between the response elements of GA and JA hormone signaling have been observed to function in an antagonistic manner switching alternatively between plant growth and defense responses (Yang et al., 2012). However, this crosstalk mechanism has not been applied to sexual development in mazie. Numerous other species are observed to be responsive to hormonal influence during sexual development (Korpelainen, 1998; Golenberg & West, 2013).

Although common, hormonal influence of sex determination is not always necessary. The persimmon *Diospyros lotus* is a tropical dioecious plant species with XY males but homomorphic sex chromosomes. Expression of *MeGI*, a homeodomain transcription factor is observed to be female specific and functions to suppress male organ development (Akagi et al., 2014). *MeGI* expression is prevented in male flowers by the tissue specific expression of *OGI*. The *OGI* gene is observed to transcribe small RNAs with complementarity to *MeGI* suggesting a form of epistatic control based on siRNA (Akagi et al., 2014). The identification of elements involved in gynoecium abortion is still undetermined.

The *Spinacia, Cucumis*, and *Diospyros* sex determination models are novel in that the mechanisms for sex determination are all based on the epistatic regulation of organ fate. In these cases, single gene activities act upon secondary target genes to trigger alternative sexual development. This implies that regulation of single genes, either by allelic segregation or by differential expression under environmental (hormone) cues can drive sexual determination. This contrasts with classic models (Charlesworth & Charlesworth, 1978) in which alternative expression of multiple genes is regulated by chromosomal segregation over generations, and must be coordinated through chromosomal linkages. Because of the necessity for alternative segregation of chromosomes with sex determining genes, heterogametic sex determination systems, and by extension, heteromorphic sex chromosomes must evolve. The *Spinacia, Cucumis,* and *Diospyros* models, because they are dependent on a single sex determining gene that acts as a switchpoint, need not suppress recombination and hence, need not evolve classic sex chromosomes (Golenberg & Freeman, 2006; Sather *et al.*, 2010; Golenberg & West, 2013; Ma & Pannell, 2016; Renner, 2016). The scarcity of degenerated sex chromosomes and sex determination genes in non-recombining

regions of such chromosomes, may hint that the single gene switchpoint model of sex determination found in *Spinacia, Cucumis,* and *Diospyros* could be a common mechanism for sex determination in plants, therefore precluding the evolution of heteromorphic sex chromosomes. Hormonal switches in monoecious species, such as *Cucumis* or *Zea*, similarly reinforce the concept that alternative gene expression can be driven by single cues and not be dependent on presence or absence of alternative sex-determining genes. Similarly, Diggle et al (Diggle, P. K. *et al.*, 2011) demonstrated that the developmental stage of alternative organ abortion or initiation in unisexual flowers within a species tends to be identical across gender, reinforcing the potential underlying process of a single switchpoint in development. A model in which male and female suppressing genes evolve and act independently would not predict such temporal coordination.

Without discrete, alternative sex organ-sterilizing loci that would necessitate non-recombination to prevent the constant regeneration of hermaphroditic or sterile offspring, there would be no selective drive for the development of heteromorphic sex chromosomes. However, this does not exclude the possibility that one chromosome of a homologous pair could function as a sex determining chromosome. Indeed, some sex determination models that are described as XY or ZW with homomorphic chromosomes (Renner, 2014; Kersten et al., 2017), may reflect the necessity of homozygosity or heterozygosity at switchpoint genes rather than genically evolving whole chromosomes. With sex determination uncoupled from degenerating chromosomal regions, we may also predict that genes involved in sex organ development and secondary sexual characteristics could be found throughout the genome and would not necessarily be anchored to any sexually segregating chromosome or region thereof. Taking the link between sexual determination and hormonal signaling into account

one might expect to find genes involved in sexual development to be responsive to the same endogenous signals and thus be expressed in coordination. Hierarchical clustering of expressed genes from RNA seq experiments will allow identification of suites of genes that are expressed in a sex specific and coordinated way (Langfelder *et al.*, 2008).

It must be emphasized that a model of sex determination based on a single epistatic, switchpoint segregating locus, whether it is related to hormonal response or to epigenetic regulation, does not supplant the chromosomal theory of sex determination regulation. Rather these should be viewed as a continuum of differing genetic sex determination systems (Golenberg & West, 2013). The existence of switchpoint sex determination systems may be a partial explanation for the paucity of examples of heteromorphic chromosomal systems in the literature, although recent evolution may also explain the difficulty in identifying neo-sex chromosomes (Bachtrog, 2011). Therefore, the absence of detected heteromorphic chromosomes cannot be inferred either to support a single locus, epistatic model or to refute an early segregating linked multi-locus model. Indeed, in spinach itself, further genomic studies may divulge the location of sex determining genes imbedded in non-recombining chromosomal regions. However, more importantly, a single locus, epistatic systems provide a rational bridge between the evolution of monoecy and dioecy commonly found within single clades (Diggle *et al.*, 2011; Renner, 2014; Ma & Pannell, 2016). As such, while some recent genome studies of the evolution of non-recombining chromosomal regions are highly informative (Ming et al., 2007; Iovene et al., 2015), it may prove more productive to use combinations of transcriptomic, reverse-genetic, and functional testing approaches to dissect the origin of sex determination in the majority of unisexual-flowering species.

**Acknowledgements**

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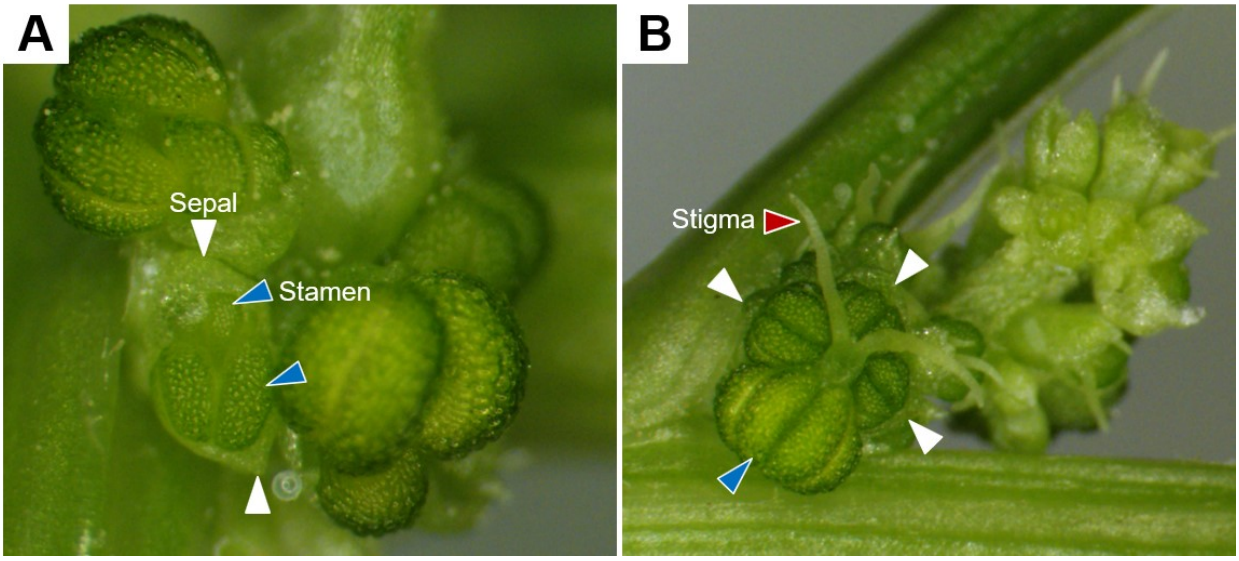
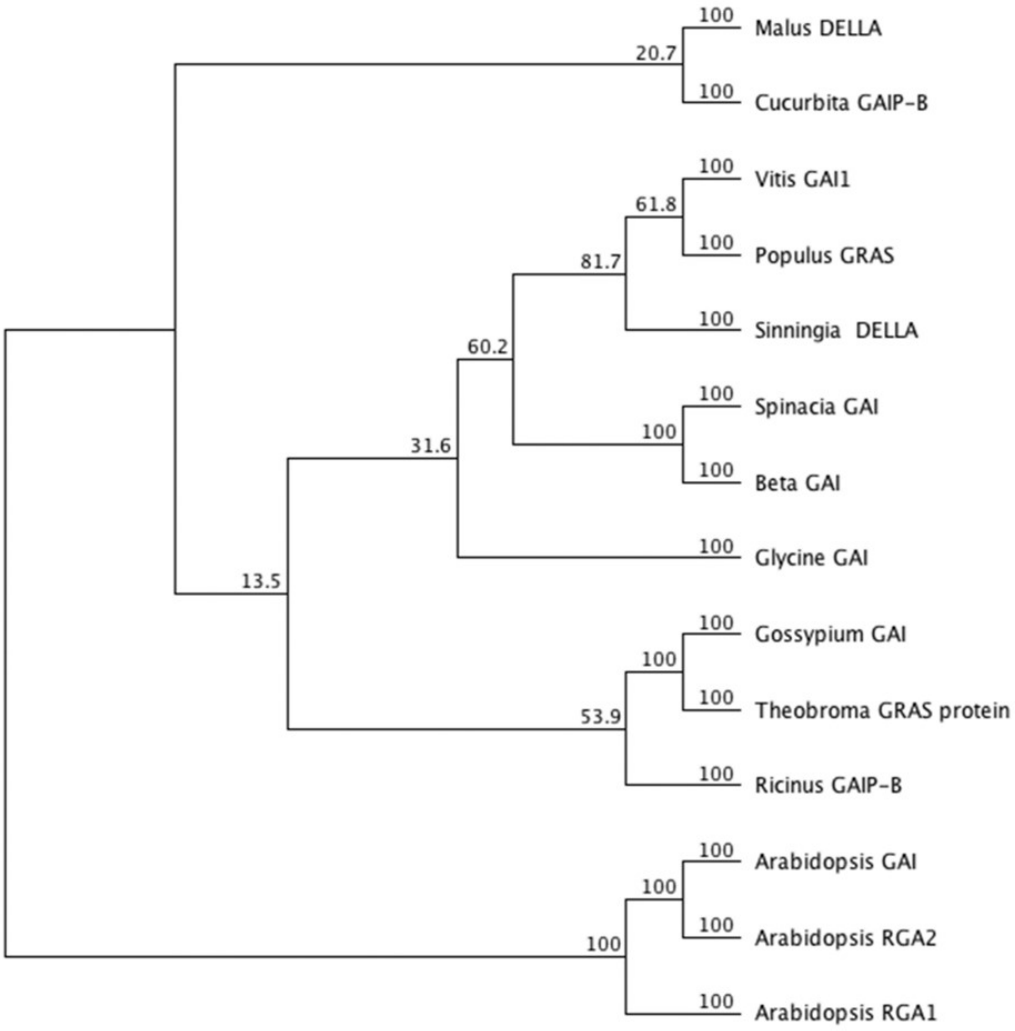
**Authors contributions**

N.W.W. and E.M.G. collaboratively planned and designed the research, performed the experiments, analyzed the data, and wrote the manuscript.

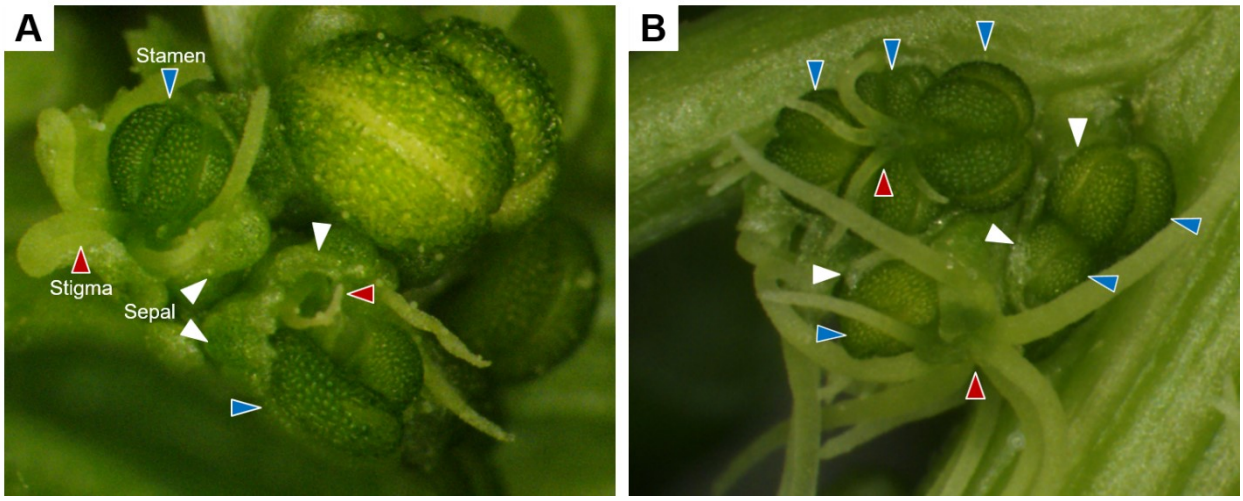



***Supplemental Figure 2.1:*** *Moderate and Severe Masculinization of Female Spinacia oleracea resulting from exogenous GA treatment.* ***(A)*** *Moderately masculinized female flower with two sepals, two stamen, and no gynoecia.* ***(B)*** *Severely masculinized female flower with four sepals, four stamen, and poorly developed pistil. White arrow = sepals, blue arrow = stamen, red arrow = stigma.*


***Supplemental Figure 2.2:*** *Moderate and Severe Feminization of Male Spinacia oleracea Flowers Resulting from PAC Application.* ***(A)*** *Moderately feminized male flower with two sepals, single stamen, and single pistil.* ***(B)*** *Severely feminized male flower with four sepals but a central pistil.* ***(C)*** *Rare example of stigma and stamen developing from the same tissue. White arrow = sepals, blue arrow = stamen, red arrow = stigma.*

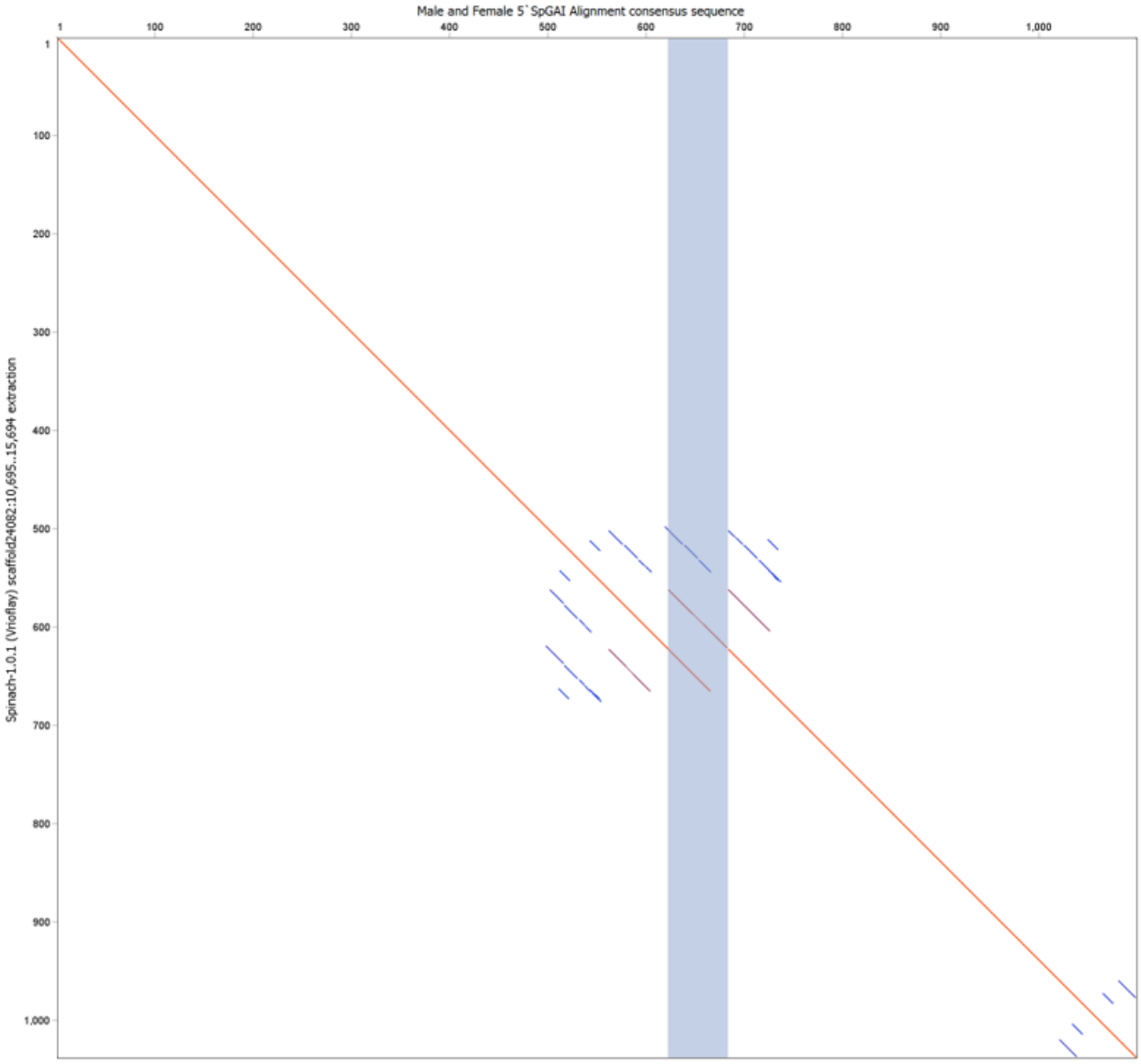



***Supplemental Figure 2.3:*** *GAI Neighbor-Joining Gene Tree Estimated from Aligned Predicted Amino Acid Sequences. Numbers on branches refer to branch support from 10,000 bootstrap resampling analyses.*




***Supplemental Figure 2.4:*** *Moderate and Severe pWSRi:GAI Knockdown Phenotypes Observed in Spinacia oleracea.* ***(A)*** *Moderate phenotype displaying two sepals, a single stamen and single pistil.* ***(B)*** *Range of phenotypes including a severely affected flower with four stamen and a central pistil (Top left), moderately affected flower with two sepals and two stamen (Middle right), and mildly affected flower with two sepals, single pistil, and single stamen (Bottom center). White arrow = sepals, blue arrow = stamen, red arrow = stigma*

***Supplemental Figure 2.5:*** *Full alignment between Spinacia oleracea cv Viroflay genomic extract and male and female 5`SpGAI sequences. Genomic reference highlighted in yellow, dots indicate agreement, dashes indicate gaps inserted into reference.*




***Supplemental Figure 2.6:*** *Dot plot of Spinacia oleracea cv America and cv Viroflay showing acluster of repetitive sequences preceding SpGAI. Blue box highlights the cv America specific 61bp insertion. Blue lines indicate short matches, red lines indicate a match length over 100bp.*

| Primer Name | Sequence | Amplicon Length |
|---|---|---|
| SpGAI qP1773F | CTGTTAGACTTCTTTGCAGG | 149bp |
| SpGAI qP1922Rev | TTGAACTCAGTGACGAATTG | |
| SpPI qP449F | CAACGATGCTTCAAGAGGAA | 156bp |
| SpqP605Rev | CTTCCCTGTTGGTTTGGTTG | |
| SpUBQ5 qP5F | CAGATTTTCGTGAAAACCC | 203bp |
| SpUBQ5 qP208Rev | TGAAGAGTTGATTCCTTCTG | |
| DELLA.514 | GANACYGTTCAYTATAAYCC | |
| DELLA.1123R | CCRTTYTCYTGSGAGTCRACSAG | |

***Supplemental Table 2.1:*** *Primer sequence and resulting amplicon length used in quantification of SpGAI and SpPI expression.*

# CHAPTER 3: IDENTIFICATION AND CHARACTERIZATION OF DIFFERENTIALLY EXPRESSED GENES IN THE UNISEXUAL FLOWERS OF *SPINACIA OLERACEA* L.

## INTRODUCTION

The morphological variation observed between individuals within a population or between tissues within an individual is often, but not exclusively, explained in terms of alternative gene expression. By carefully grouping individuals from a population or tissues from an individual then generating a transcriptome it is reasonable to expect that transcripts identified as differentially expressed between the groups contribute to the morphological differences in question. However, the identified transcripts actually would be a mixture of those functionally related to the morphology of interest, correlated to but not necessary for morphological differentiation, and false positives. In order to distinguish among these possibilities, it will be helpful to put the identified transcripts into a biological context.

Alteration of expression patterns can portend developmental events such as embryo organization (Mayer *et al.*, 1993), cell lineage (Haecker *et al.*, 2004), and organ identity (Meyerowitz *et al.*, 1991). Genes involved in the aforementioned processes tend to be highly conserved transcription factors that initiate a cascade of gene expression. The plant transcription factor LEAFY is a good example as it controls the identity of the floral meristem (Weigel *et al.*, 1992), is observed to be highly conserved among plants in both sequence (Maizel *et al.*, 2005) and function (Chujo *et al.*, 2003) and alters the expression of numerous genes (William *et al.*, 2004). Identifying genes downstream of important transcriptional regulators through traditional methods, such as microarray analysis, can be difficult, time consuming, expensive, and/or not available in the species of interest. However, recent advancements in massively parallel sequencing technologies allow investigators to identify

large numbers of transcripts that are correlated with a morphology of interest. Similar to traditional methods next gen sequencing only identifies correlated transcripts and does not inform directly about the function of said transcripts.

Dioecious angiosperms, and spinach in particular, are ideal models to investigate the relationship between alternative gene expression and morphological development. Dioecious species can be grouped by sex with relative ease and the differences in gene expression between the groups is therefore expected to reflect unisexual development. Many dioecious species, including spinach, are sex labile and with proper stimuli an otherwise male individual can produce female flowers and vice versa (Korpelainen, 1998). This indicates that unisexual development hinges on the proper expression of male or female gene cohorts. Spinach is a particularly apt model as the differences between male and female vegetative growth is minimal while most morphological differences are observed after the transition to flowering. Sampling the inflorescences would presumably maximize observation of differential gene expression related to unisexual morphology and observations of *in situ* expression will help put the sequencing data into biological context.

Spinach flowers do not produce petals, developing only sepals and one type of reproductive organ. The peripheral whorl is fated to become the sepals while the interior whorl will develop the androecium or gynoecium. The first organ to develop from the floral meristem is the sepals, the number of which is sex specific (Sather, D Noah *et al.*, 2005). The male flower develops four sepals that remain separate from each other while females produce two sepals that often fuse along a portion of their margin. Male flowers develop four stamens in a phyllotaxic pattern while the central area of the meristem flattens. The female flower produces a single carpel from the central meristem that is enveloped by the

sepals with only the stigma exposed (Sather, D Noah *et al.*, 2005). Both sexes produce small clusters of flowers in the leaf axil however, in some males the leaf is significantly reduced in size or absent in the upper portion of the flowering branches (Rosa, 1925).

To characterize genes differentially expressed in unisexual spinach flowers we produced a transcriptome from male and female inflorescences. Transcripts that were found to be correlated to sex were analyzed with BLAST and from these data candidate genes were selected for further investigation. Numerous genes identified as differentially expressed via transcriptome analysis were confirmed with qRT-PCR of inflorescence tissue and the expression pattern of a select few genes were observed with LAMP mediated *in situ* observation.

## METHODS

### RNA isolation, library construction, and transcriptome sequencing

Total RNA of inflorescence samples was isolated and purified using a RNeasy Mini Kit (Qiagen, Hilden Germany) according to the manufactures' protocols. RNA integrity was evaluated using an Agilent 2100. RNAseq libraries were made using ScriptSeq kit (Epicenter) following the manufacturers' protocol. Poly (A) mRNA was isolated from a total input of 5µg of RNA using oligo (dT)-attached magnetic beads according to the manufactures' instruction (Illumina, USA). Approximately 50ng of purified RNA was fragmented according to protocol with the exception that fragmentation at 85°C was reduced from the recommended 5 minutes to 30seconds. First strand cDNA was made using random hexamers, then tagged and amplified for 17 cycles using barcoded primers. Samples sent to RTSF Genomics Core at Michigan State University (Lansing, MI) and analyzed with Illumina HiSeq 2000. Raw reads were trimmed, removing sequencing adapters and low-quality bases

using Trimmomatic (Bolger *et al.*, 2014) with default settings. Contigs were generated *de novo* using IDBA-tran (Leung *et al.*, 2013; Peng *et al.*, 2013) with settings for minimum contig size of 200 nucleotides. Differential expression was assessed using RSEM v1.2.24 (Li & Dewey, 2011) and EBSeq v1.11.1 (Leng *et al.*, 2013). Annotation of select differentially expressed genes were produced using BLAST2GO (Conesa & Götz, 2008; Götz *et al.*, 2008).

**qRT-PCR of Differentially Expressed Genes**

Inflorescence RNA from male and female individuals were extracted and purified using plant RNeasy Mini Kit from Qiagen. 5µg of RNA was used as template to create first strand cDNA using ClonTech RNA to cDNA EcoDry Premix (Takara Bio USA Inc. CA, USA). qPCR was performed on an Agilent Technologies Mx3000P machine (Agilent Technologies Inc. CA, USA) using iTaq Universal SYBR Green Supermix buffer (BioRad CA, USA). Primer sequence information can be found in Table S1. Amplification conditions were as follows: denaturation at 96°C for 10min, then 40 cycles composed of 30s denaturing at 96°C, annealing at 55°C for 30s, extension at 72°C for 30s. Melting point profiles were examined to confirm that single PCR products were produced. Expression values were determined with the ΔΔCt method using Ubiquitin 5 (UBQ5) as the internal reference gene (Gutiérrez-Aguirre *et al.*, 2008). For all genes analyzed female expression values were set to 1.0 as the calibrator sample and male expression reported relative to that value.

**Tissue preparation and LAMP in situ detection**

Spinach inflorescences were harvested during bolting but before anther dehiscence or successful pollination. Tissue was fixed, embedded in paraffin wax, sectioned and prepared for LAMP mediated detection following Sather et. al. 2010, Podushkina et. al. 2019. Briefly, inflorescence tissue was fixed for 10-14 hourss at 4°C in Formaldehyde Alcohol

Acetic acid (FAA) solution, dehydrated through an ascending alcohol series (70%, 95%, 100%), followed by Histo-Clear (National Diagnostics) washes. The tissues were imbedded in paraffin at 60°C with multiple exchanges of liquid paraffin over a 6 hour time period. 8µm tissue sections were adhered to Matsunami TruBOND 380 slides by baking overnight at 45°C and carefully stored at room temperature.

To prepare the tissue for LAMP mediated *in situ* gene detection the paraffin wax was removed with Histo-Clear washes and rehydrated in a descending alcohol series (100%, 95%, 70%, 50%), rinsed in nuclease free water, and equilibrated in tris buffered saline (TBS) solution. Samples were then incubated in Proteinase K (20 µg/mL) at 37°C for 20 minutes and thoroughly washed in TBS. The sections were treated with DNase I (1U) at 37°C for 45min. A 30µL LAMP reaction (Warm LAMP, New England Biolabs) with gene specific primers (Table S3) and 0.15µL digoxygenin-11-dUTP (25nmol) was applied drop-wise directly onto tissue samples and incubated at 65°C for 45min in a humidity chamber. Every 7min the chamber was gently rocked to encourage even distribution of the reaction solution over the tissues. Negative control reactions were assembled with water replacing the gene specific primers in the reaction mix. Slides were then washed in malic acid buffer and incubated in 1% dry milk blocking buffer before being challenged with anti-DIG antibody conjugated with alkaline phosphatase. Slides are then thoroughly washed in malic acid buffer and incubated in Roche (Basel, SZ) NBT/BCIP solution until precipitate is visible, usually within 30 minutes.

## RESULTS

### Transcriptome Production and Analysis

Floral RNA was harvested, purified, and sequenced using Illumina HiSeq 2000. The resulting fragments were trimmed with trimmomatic using default settings yielding 13,456,406 and 32,976,700 reads for male and female libraries respectively. The reads were assembled into contigs using IDBA-tran with default settings producing 122,322 male contigs and 442,674 female contigs. The data from both genders were combined to generate a single library of contigs. This resulted in 466,410 contigs. The gender specific reads were then mapped to the combined spinach floral transcriptome library and were then analyzed with RSEM (Ver. 1.2.24) and EBseq (Ver.1.11.1) to identify differentially expressed contigs (Table S2).

A total of 165 contigs were identified with higher than 0.95 posterior probability of differential expression. The vast majority of these, 158 were upregulated in male inflorescence tissue. Only 7 were found to be overexpressed in female tissue. Of these 165, 88 were identified with BLAST (Table S1). These included genes involved in stamen organ identity (*SpAPETALLA3, SpPISTILLATA*) that had previously been identified as having male-specific gene expression (Pfent, Catherine *et al.*, 2005). Among the remaining male-biased expressed, multiple clusters could be readily identified based on functionality (Table 1).

**Selected Spinach Male Overexpressed Genes**

| Name | Post. Prob. Differential Expression |
|---|---|
| **Stamen Organ Determination Or Function** | |
| Mago Nashi Homolog (Negative Regulator Of Transposition) (Oocyte Formation) | 1 |
| Nac Transcription Factor 25-Like (Anther And Pollen Development) | 0.999999744 |
| Aborted Microspores | 0.999936366 |
| Pistillata (Pi) Gene | 0.999984418 |
| Apetala3 (Ap3) Gene, | 0.987646576 |
| Chalcone Synthase (Pollen Fertility) | 0.999661087 |
| Eceriferum 3 (Sporopollenin Production) | 0.999273709 |
| S-Adenosylmethionine Decarboxylase Proenzyme-Like (Required For Male Fertility, Pollen Viability) | 0.997995480 |
| **Hormone Pathways** | |
| **Auxin** | |
| Multicaulis Auxin Efflux Carrier | 0.995552243 |
| Indole-3-Acetic Acid-Amido Synthetase | 0.982981328 |
| **Ethylene** | |
| 1-Aminocyclopropane-1-Carboxylate Oxidase | 1 |
| **GA** | |
| Calcineurin B-Like Protein (Responds To Ga, Cold Stress) | 0.969713251 |
| Cold Acclimation Protein (Cap160) Gene | 0.994863393 |
| Abc Transporter G Family Member 28 (Cold Stress Response) | 0.999896892 |
| **Abscisic Acid** | |
| Abscisic Acid 8'-Hydroxylase 1-Like | 0.993674613 |
| **Stress Response** | |
| Cation/H(+) Antiporter 15 (Salinity, Osmotic Stress) | 0.999999968 |
| Betaine Aldehyde Dehydrogenase (Badh) Gene, (Salinity, Osmotic Stress) | 1 |
| Cytosolic Heat Shock 70 Protein (Hsc70-3alpha) Gene, | 0.999107078 |
| Desiccation-Related Protein Pcc13-62-Like | 0.993413717 |
| Lrr Receptor-Like Serine/Threonine-Protein Kinase Fls2 (Bacterial Resistance) | 0.993857872 |
| Calmodulin-Like Protein 7 (Light Induction Growth) | 0.974797737 |
| **Cell Wall Dynamics** | |
| Pectin Methyltransferase Qua2 | 0.999999995 |
| Galacturonosyltransferase-Like 4 | 0.998959413 |
| Pectinesterase/Pectinesterase Inhibitor 21 | 0.999997858 |
| Pectinesterase/Pectinesterase Inhibitor 51 | 0.998957890 |
| Putative Expansin-A26 | 0.990706536 |
| Wat1-Related Protein At1g43650-Like (Secondary Wall Deposition) | 0.989166133 |
| **Miscellaneous** | |
| Sex Male 5s Ribosomal Rna Genes | 0.999561825 |
| FBD-Associated F-Box Protein | 0.996922829 |

***Table 3.1 –*** *Male biased BLAST identified genes*

**RT-qPCR Analysis of Unisexual Inflorescences**

In order to corroborate the transcriptome data, we investigated the expression of several candidate genes. Multiple inflorescences from an individual were harvest, pooled, and RNA was extracted from multiple male and female individuals. Expression of selected genes was observed via qRT-PCR using the ΔΔCt method with the female samples as the calibrator and *SpUBIQUITIN 5* as the internal reference gene. The expression paaterns of *Aborted Microspores* (*SpAMS*) and *Abscisic Acid 8`-Hydroxylase 1-Like* (*SpABA*) were observed to be significantly overexpressed in male tissue ($p<0.001$). Likewise, *NAC Transcription Factor 25-Like* (*SpNAC*) and *Indol-3-Acetic Acid-Amido Synthetase* (*SpIAA*) are highly expressed in male inflorescence ($p<0.01$). The mean expressions for *Adenine phosphoribosyltransferase 1-like* (*APT*), *Expansin A26* (*Expan*), *Galacturonosyltransferase-Like 4* (*GAUT*), and *Pectinesterase/Pectinesterse inhibitor 51* (*Pect*) were elevated in the male samples however, this elevation does not appear significant (Figure 3.1).

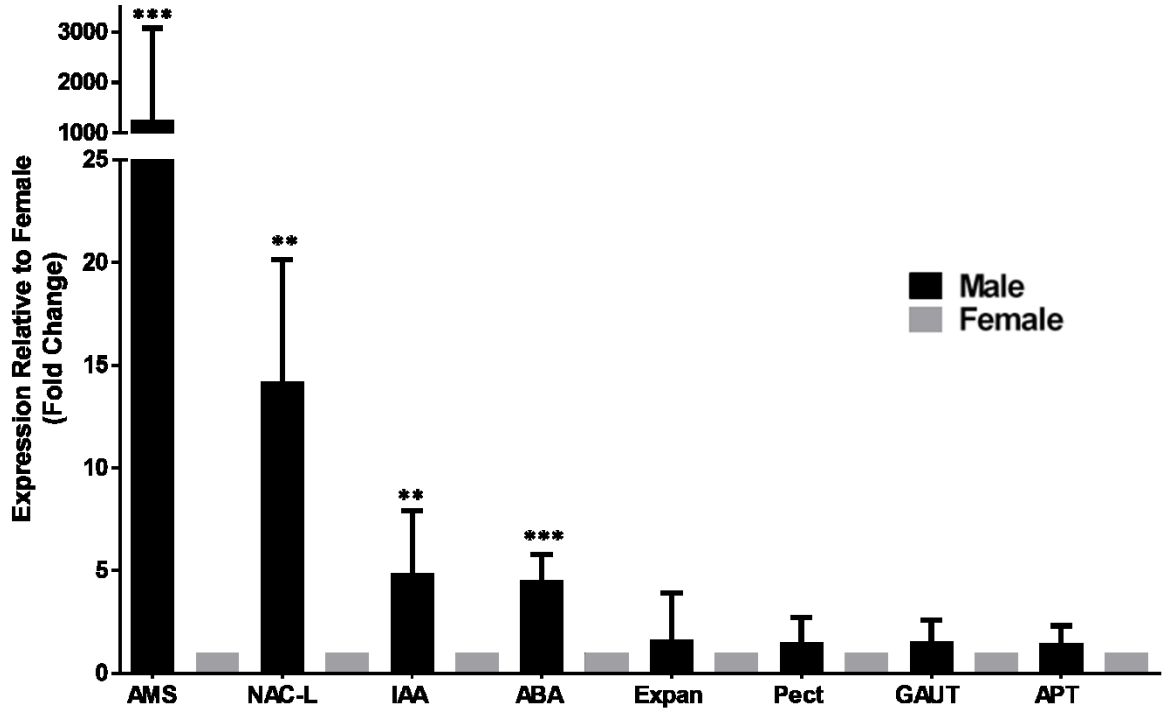


***Figure 3.1 –*** *Relative expression of select genes overrepresented in male samples from RNAseq analysis. Genes identified as differentially expressed through in silico techniques were analyzed via qPCR. Expression calculated using the ΔΔCt method setting the female data to 1 as the calibrator sample. Error bars indicate SD (n = 5 independent samples), asterisks represent p<0.001 (***) and p<0.01 (**) from one-way ANOVA analysis. Male samples (n=3) in black bars, female samples (n=3) in grey bars.*

Similarly, the mean expression of *Elongation factor 1-alpha-like* (*EF1*) was reduced in males but otherwise not significantly different from female expression levels. Expressions of *F-box/LRR-repeat At3g26922-like* (*Fbox*) as well as *Female Unknown 1-4* (*FemUn1-4*) were significantly reduced in all male samples (p<0.001), with *FemUn3* being the least expressed of them all. The elevated female expression of *SpFbox* was unexpected as the differential expression report indicated increased male expression (Figure 3.2).

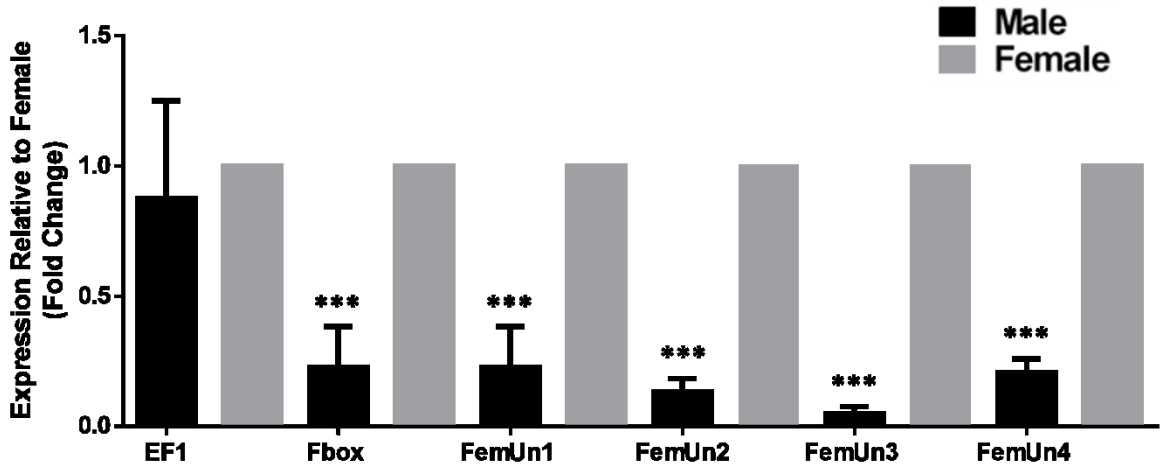



***Figure 3.2*** *– Relative expression of select genes overrepresented in female samples in RNAseq analysis. Genes identified as differentially expressed through in silico techniques were analyzed via qPCR. Expression calculated using the ΔΔCt method setting the female data to 1 as the calibrator sample. Error bars indicate SD (n = 5 independent samples), asterisks represent p<0.001 (***) from one-way ANOVA analysis. Male samples (n=3) in black bars, female samples (n=3) in grey bars.*

**Aborted Microspores *in situ***

*AMS* (*ABORTED* MICROSPORES) is a MYC class transcription factor that is required for proper androecium development. When mutated, premature tapetum and microspore degeneration, and reduced stamen filament growth were observed in *A. thaliana* (Sorensen *et al.*, 2003). *SpAMS* is observed to have 60.6% sequence similarity to *AtAMS* (Supplemental Figure 3.1). Previously in our laboratory, we observed *SpAMS* expression in microsporangial and tapetal tissues in spinach (Podushkina *et al.*, 2019) using fluorescently labeled primers. Instead of gene specific fluorescent primers, we used unlabeled primers and incorporated Digoxygenin-11-dUTP bases into the LAMP reaction which was then visualized with an alkaline phosphatase conjugated anti-digoxygenin antibody and NBT/BCIP substrate. Male tissue visualized are cross-sections through early anthers (Figure 3.3a, 3.3d) and anthers later in development (Figure 3.3b) while the female inflorescence tissues are shown in longitudinal section (Figure 3.3c). *SpAMS* expression is strong in early anther tissue, both in the L1 layer and in the internal premicrosporangial tissue (Figure 3.3a).

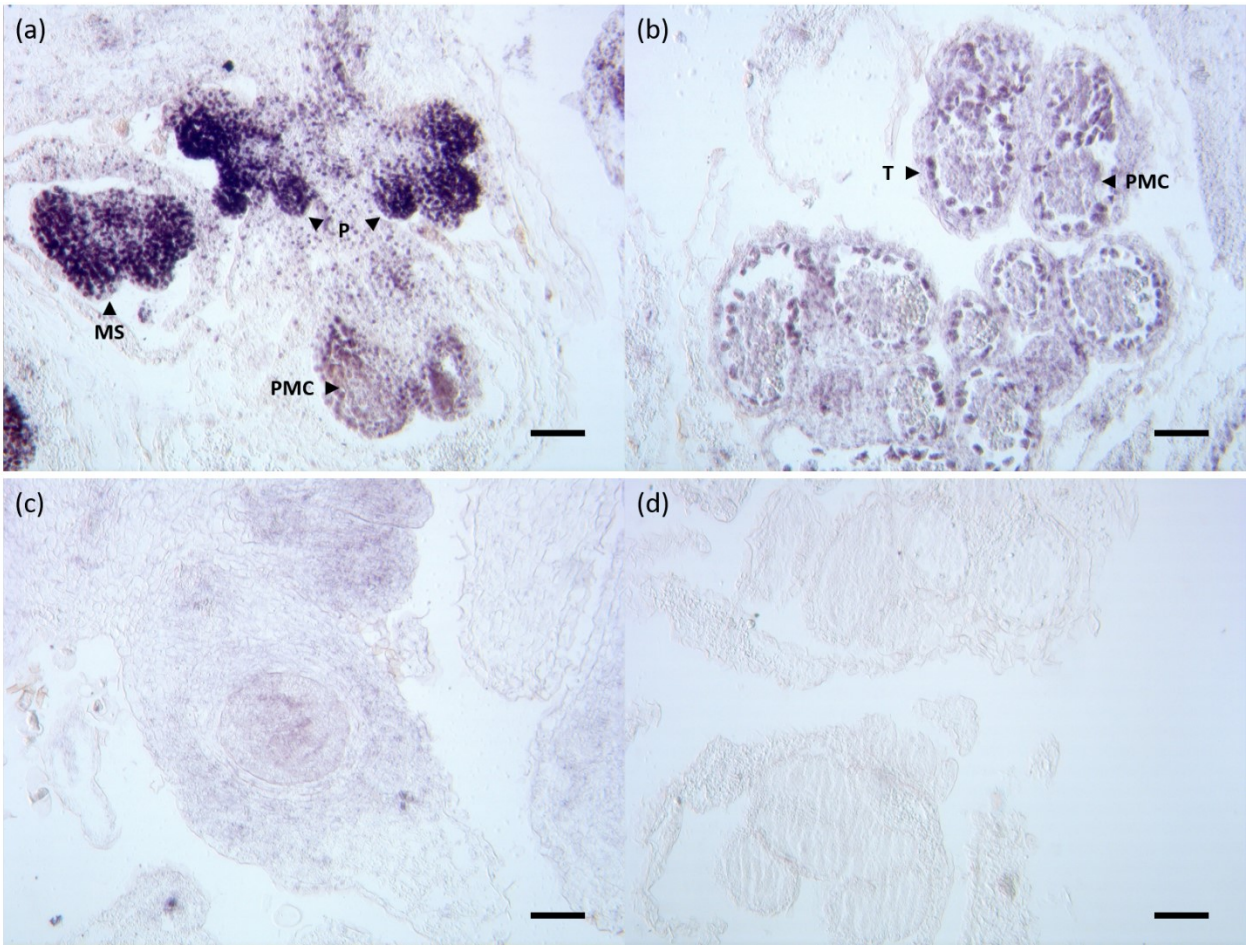



***Figure 3.3 –*** *SpAMS expression in male and female inflorescences. Shoot apicies were harvest before bolting, sectioned and gene expression was visualized with a colorimeteric LAMP reaction. (a) Male inflorescence cross-section with expression observed in primordia (P), microsporoangia (MS), and early pollen mother cells (PMC). (b) Male cross-section with tapetum (T) and pollen mother cells stained lightly. (c) Female inflorescence longitudinal section with no significant expression observed. (d) Male cross-section negative control with no observed signal. Scale bars indicate 100μm*

In more developed flowers, the expression level decreases and is not strong throughout the anther (Figure 3.3a arrow). In more mature male flowers, *SpAMS* is strongly expressed in the tapetal tissue (Figure 3.3b) and present but weaker in later stages as pollen mother cells develop (Figure 3.3b). In contrast, we detected no *SpAMS* expression in the inflorescences of females (Figure 3.3c). As a negative control to test for non-specific binding

of digoxygenein-11-dUTP or anti-digoxygenin conjugates, male tissue sections were challenged with LAMP reaction mix without gene specific primers resulting in no detectable signal in the male negative controls. These data are consistent with previous observations (Podushkina *et al.*, 2019) and suggest a role for *SpAMS* in early anther development and later pollen development through the expression tapetal and microsporangial tissues.

**Mago Nashi *in situ***

Transcriptome analysis identified a highly expressed, male specific transcript predicted to be similar to *MAGO NASHI* (*mago*). Initially found in *Drosophila (Boswell et al., 1991)*, *mago* has also been found in *C. elegans* and humans (Zhao *et al.*, 1998; Li *et al.*, 2000; Zhao *et al.*, 2000). A *mago* ortholog was identified in rice (*Oryza sativa*) and expression was observed in developing root, leaf and seed tissue via RNA and protein blot analysis (Swidzinski *et al.*, 2001). The spinach ortholog *SpMago* is observed to have 80.5% sequence similarity to *AtMago* (Supplemental Figure 3.2) whose function in *A. thaliana* was investigated through RNAi. Among other growth defects, microspore formation, pollen production and germination rates, and seed development was compromised in the RNAi-*AtMago* plants supporting the importance of *AtMago* function in reproduction (Park *et al.*, 2009). To investigate the expression pattern of *SpMago*, we performed LAMP based *in situ* with DIG labeled dUTP. The male secondary inflorescence was sectioned longitudinally with primordia clearly visable (Figure 3.4a). *SpMago* is strongly expressed in very early male inflorescence tissue found in the axils of leaves with less expression observed in the neighboring vegetative tissue (Figure 3.4a). Expression is also observed throughout microsporangial tissue but not in subtending filamentous tissue nor sepal tissue (Figure 3.4b). In more mature flowers, there is distinct *SpMago* expression in the tapetum but less

expression in pollen mother cells (Figure 3.4a arrow). No detectable expression was observed in a longitudinal section of a well-developed female ovary (Figure 3.4c). We observed no staining on male tissues detected without gene specific primers as our negative control (Figure 3.4d). These data suggest *SpMago* may function in meristem organization and microspore development similar to the *A. thaliana* ortholog.

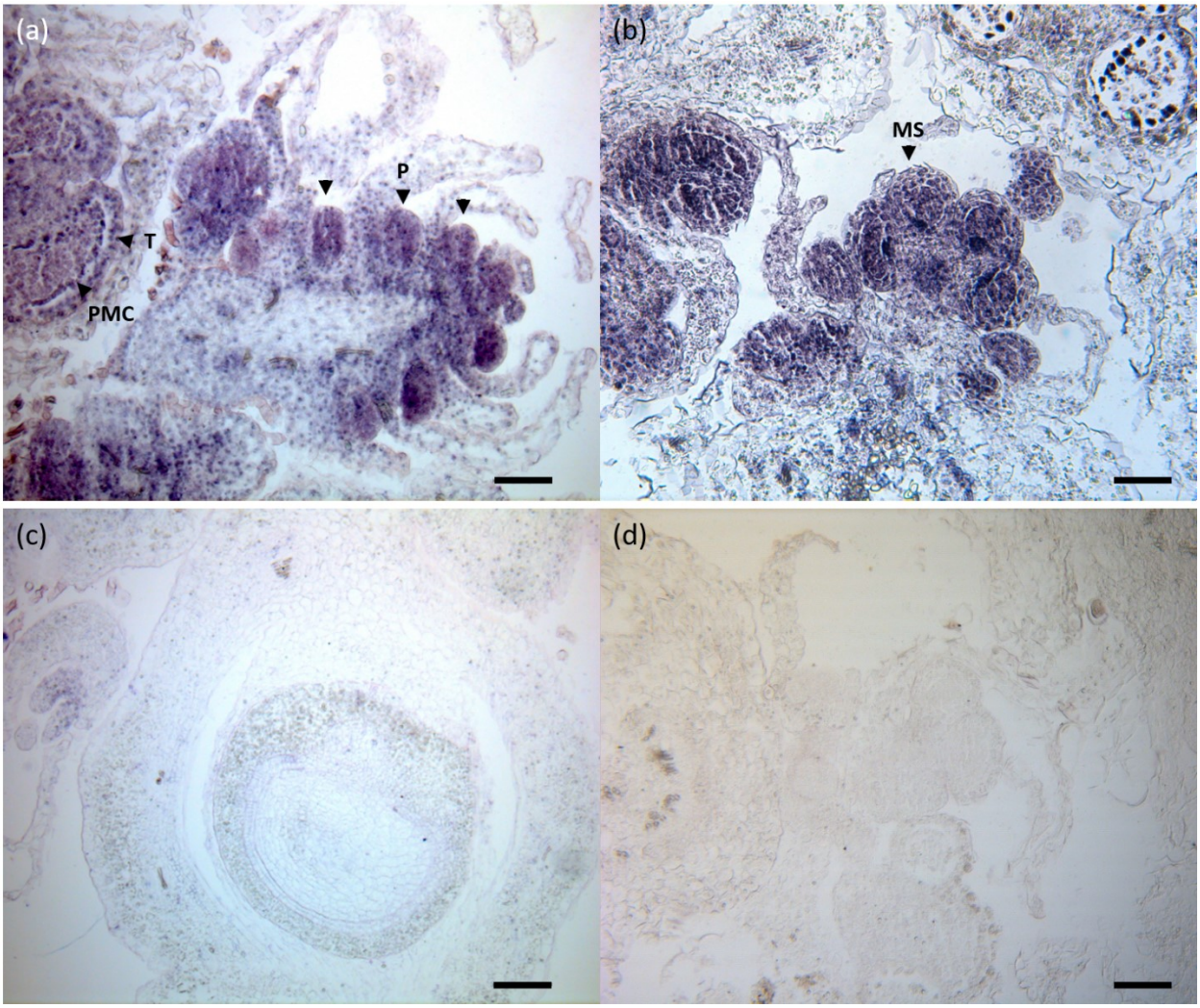



***Figure 3.4*** *– SpMagoNashi expression in male and female inflorescences. Shoot apicies were harvest before bolting, sectioned and gene expression was visualized with a colorimeteric LAMP reaction. (a) Male secondary inflorescence longitudinal section with expression observed in primordia (P) and to the left a cross section of an anther with expression in the tapetum (T) and pollen mother cells (PMC). (b) Male cross section with expression observed in microsporangia (MS). (c) Female inflorescence*

*longitudinal section with no significant expression observed. (d) Male cross section negative control with no observed signal. Scale bars indicate 100µm.*

**Fbox *in situ***

Differential expression analysis of unisexual inflorescences identified one gene that contained F-box domains and leucine-rich repeats that was highly expressed in females. This predicted gene aligns only 50.3% with an *A. thaliana* predicted F-box/LRR protein At3G26922 however, when aligned to *Beta vulgaris*, a close relative of spinach, alignment was 81.9% to the predicted F-box/LRR-repeat protein XM_019247200.1 (Supplemental Figure 3.3a, 3.3b) F-box proteins are understood to function as components of the SCF ubiquitin-ligase complexes (so named for the proteins in the complex, Skp I, Cullin, and an F-box protein), where the F-box protein binds the target for ubiquitin-mediated proteolysis (Gray *et al.*, 2002; Risseeuw *et al.*, 2003). There are 694 F-box proteins in *A. thaliana* (Gagne *et al.*, 2002) and some are known to be involved in hormone signal transduction, specifically in response to GA signaling (McGinnis *et al.*, 2003; Dill *et al.*, 2004; Fu *et al.*, 2004) which is of critical importance to sex determination in spinach (West & Golenberg, 2018). To characterize gene expression, LAMP mediated *in situ* gene amplification was performed on male in female inflorescence tissue. *SpFbox* expression was observed in very early flower primordia but not surrounding vegetative tissue (Figure 3.5a). In later stages of male development *SpFbox* is highly expressed in early microsporangial tissue but is reduced as pollen mother cells develop and is eventually restricted to the tapetum as development progresses (Figure 3.5b, 3.5a arrow). Early in female development *SpFbox* expression is observed throughout the ovary with heavy precipitate at the distal portion of the integuments and in the early nucellus (Figure 3.5d). As gynoecial development continues

this expression is restricted to integuments and funiculus with expression reduced in the nucellus and ovary walls (Figure 3.5e). As a negative control, male and female tissues were challenged with LAMP reaction mix without gene specific primers, no signal was detected (Figure 3.5c, 3.5f). The expression of *SpFbox* in males appears to decrease during development while in females expression is at higher levels and generally expressed in early tissue with expression then being restricted to specific tissues within the maturing ovary.

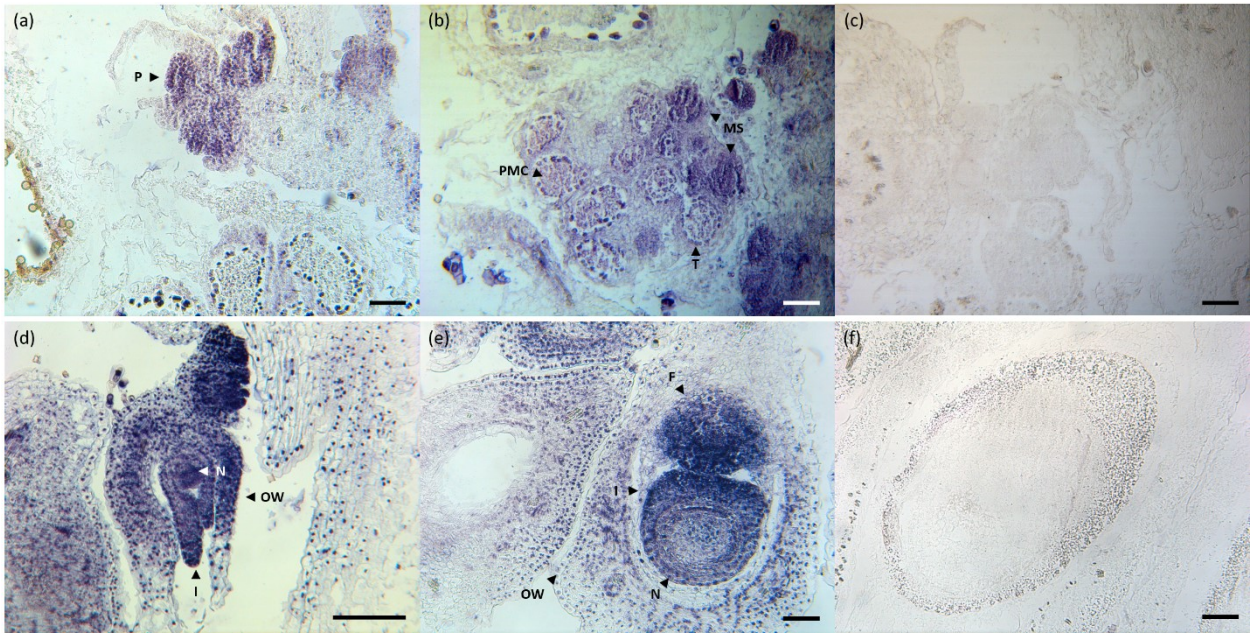



***Figure 3.5*** *– SpFbox expression in male and female inflorescences. Shoot apicies were harvest before bolting, sectioned and gene expression was visualized with a colorimeteric LAMP reaction. (a) Male inflorescence longitutinal section with expression observed in primordia (P). (b) Male cross section with expression observed in microsporangial tissue (MS) and tapetum (T) but less signal from pollen mother cells (PMC). (c) Male cross section negative control with no observed signal. (d) 20x magnification of early female inflorescence longitudinal section signal detected in the nucellus (N), integuments (I) and ovary wall (OW). (e) Female inflorescence longitudinal section with strong signal from the integuments and funiculus (F) but less signal from the nucellus and ovary walls. Scale bars indicate 100µm.*

**Female Unknown 3 *in situ***

A number of transcripts found to be overrepresented in female transcriptome samples could not be identified through BLAST analysis, one such transcript is *SpFemaleUnknown3* (*SpFemUn3*). The expression of *SpFemUn3* was observed to be essentially non-existent in male inflorescence tissues as determined by qRT-PCR (Figure 3.2). To characterize the expression pattern of *SpFemUn3* we performed LAMP mediated *in situ* analysis on male and female inflorescences. In a longitudinal section of the apical region of female inflorescence shows expression of *SpFemUn3* was isolated to pre-gynoecial tissue while being absent or reduced in neighboring vegetative tissue (Figure 3.6a). In the ovary, *SpFemUn3* strong expression was restricted to the nucellus and funiculus while weaker signal was detected in the outermost layer of integument tissue. No signal was observed in the ovary walls (Figure 3.6b). In contrast, no significant expression was observed in male inflorescences at any stage (Figure 3.6c). Likewise, female tissue challenged with a LAMP negative control reaction without gene specific primers showed no evidence of staining (Figure 3.6d). Consistent with qRT-PCR data *SpFemUn3* is female specific and is observed very early in gynoecium development as well as during ovary maturation.

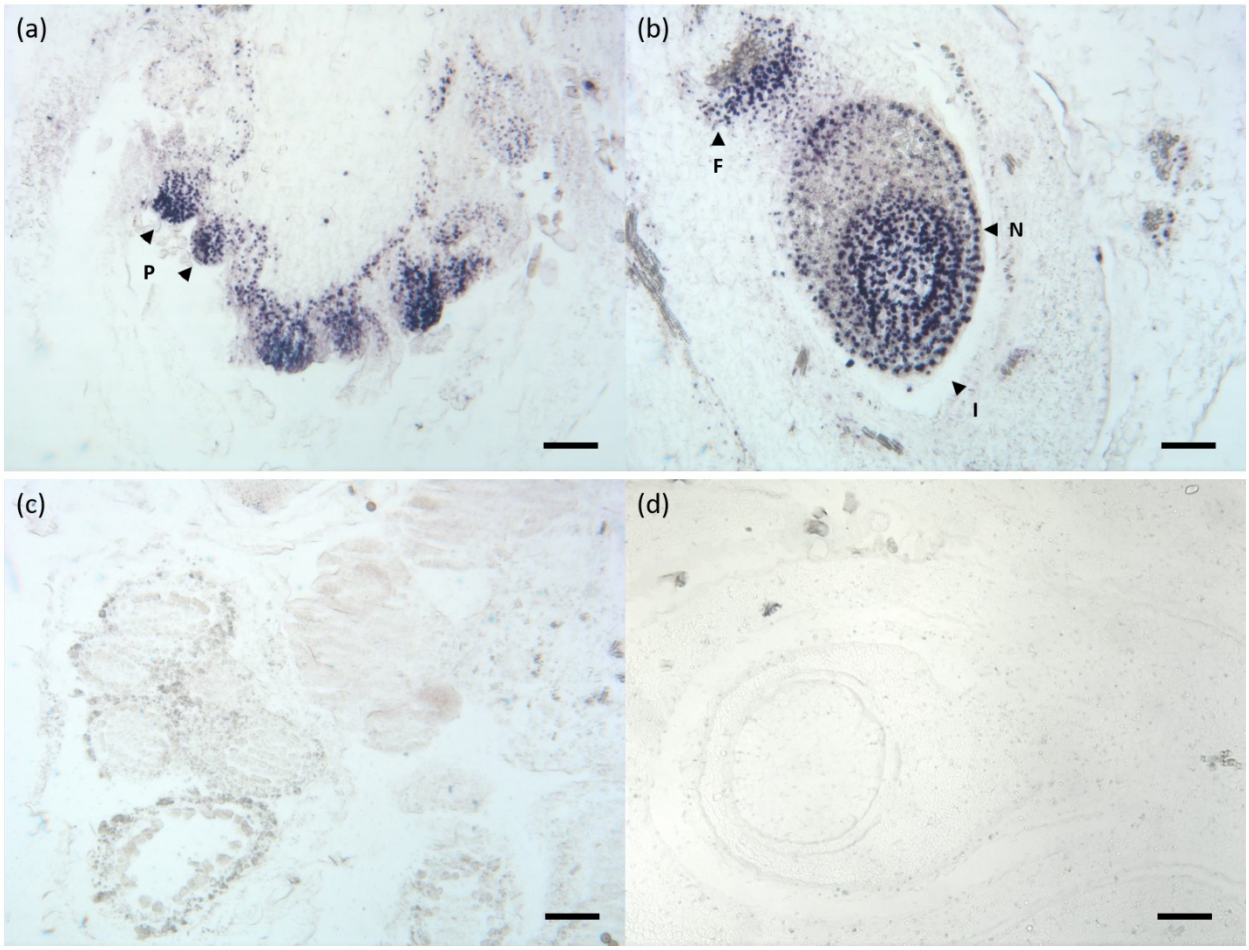



***Figure 3.6*** *– SpFemaleUnknown3 expression in male and female inflorescences. Shoot apicies were harvest before bolting, sectioned and gene expression was visualized with a colorimeteric LAMP reaction. (a) Female inflorescence meristem longitudinal section with expression observed in primordia (P). (b) Female ovary longitudinal section with signal detected in the nucellus (N) distal portion of the integuments (I) and slight expression detected in the funiculus (F). (c) Male inflorescence cross section with no detectable signal from any tissue. (d) Female ovary negative control with no detectable signal. Scale bars indicate 100µm.*

**Pectinesterase *in situ***

Overrepresentation of *SpPectinesterase/Pectinesterase Inhibitor 51* (*SpPect*) in the transcriptome analysis was not corroborated by our qRT-PCR observations. To elucidate the discrepancy *SpPect* expression was analyzed *in situ*. In a longitudinal section of a male

inflorescence we observe signal associated with vascular tissues and strong signal in early male flowers, at later stages signal is seen in stamen tissue but not in sepals (Figure 3.7a). Without gene specific primers no signal was observed in a cross section of male tissue (Figure 3.7b). A similar expression pattern was observed in a longitudinal section of female inflorescence. Signal was observed in vascular tissue and early flowers were strongly stained, as development continues signal is detected in the nucellus and proximal region of the ovary wall (Figure 3.7c).

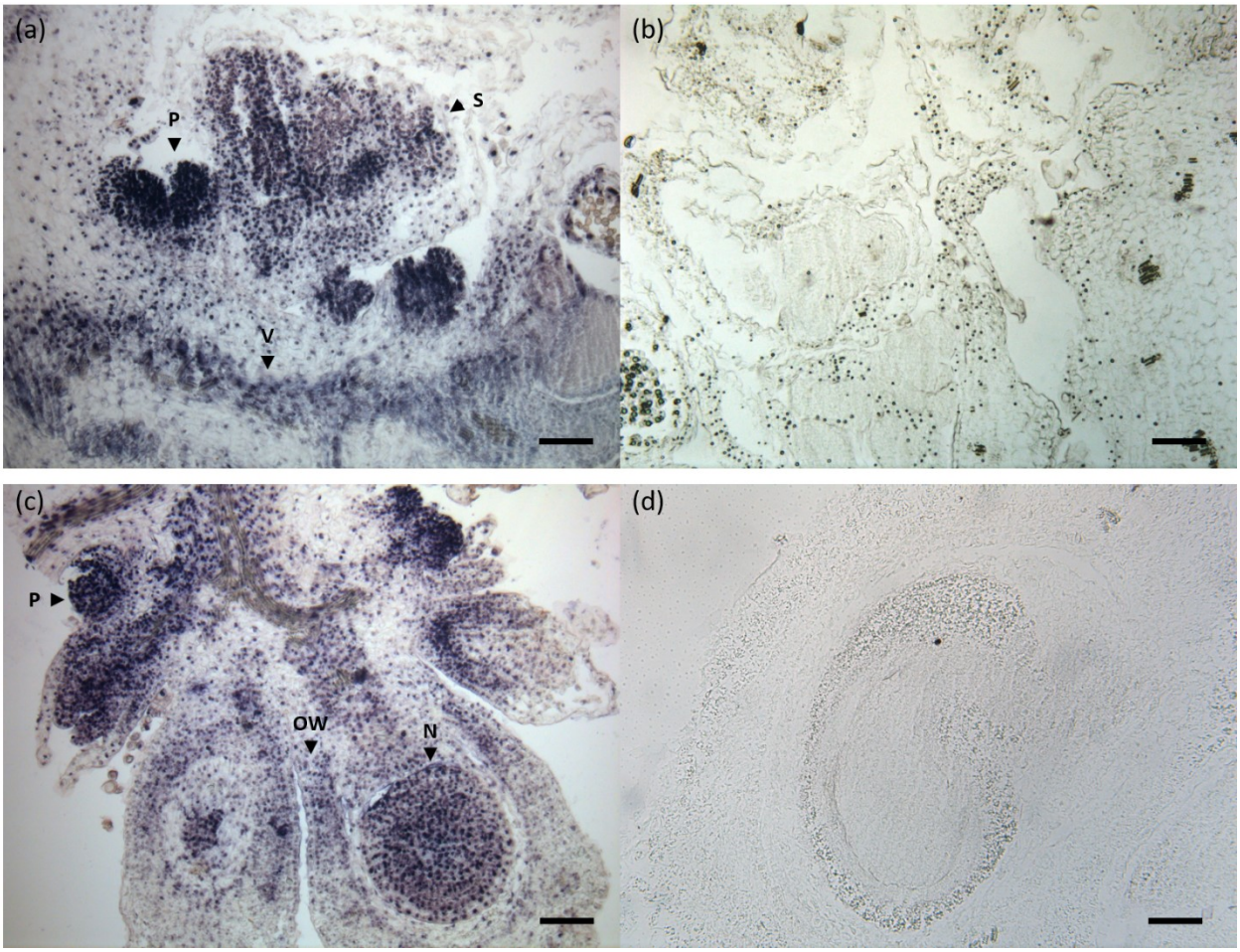



***Figure 3.7*** *– SpPectinesterase expression in male and female inflorescences. Shoot apicies were harvest before bolting, sectioned and gene expression was visualized with a colorimeteric LAMP reaction. (a) Longitudinal section of male inflorescence with staining detected in early male primordia (P), throughout the stamen (S), and light staining near vascular tissue (V). (b) Cross section of male negative*

*control, no observed signal. (c) Longitudinal section of female inflorescence, heavy expression observed in primordia and throughout the early flower as well as near vascular tissue, at later stages expression restricted to proximal portion of ovary wall (OW) and nucellus (N). (d) Female ovary negative control with no detectable signal. Scale bars indicate 100µm.*

## DISCUSSION

Next generation sequencing technologies yield massive data libraries that allow investigators to correlate sequencing data to biological phenomenon. An intriguing topic of study that leverages big data is the elucidation of unisexual development in plants (Harkess *et al.*, 2015; Du *et al.*, 2016; Mei *et al.*, 2017; Fu *et al.*, 2018). Without additional biological context for identified genes downstream, functional analysis could be significantly hampered by cryptic mutant phenotypes and futile pursuit of correlated but ultimately non-critical genes. In this study we generated transcriptome libraries of inflorescences from male and female spinach individuals and produced a list of 165 differentially expressed transcripts. To put these into biological context we performed LAMP mediated *in situ* analysis of select candidate genes and report here their expression patterns.

We began our survey with genes for which expression or function was observed in other species. The function of *A. thaliana mago* was determined via RNAi and resulted in plants of diminutive size with defects in shoot and root meristem organization as well as pollen and embryo development (Park *et al.*, 2009). The expression pattern of spinach *mago* occurred within tissue congruent with function observed in *A. thaliana* (Figure 3.4) however, our investigation offers more precise expression data than previous reports (Swidzinski *et al.*, 2001). The expression pattern of *SpFemUn3* is of interest as the transcriptome analysis

and RT-qPCR data are in agreement with this gene's over representation in female samples. However, megaBLAST searches yielded no results thus no predicted function could be ascertained. LAMP based *in situ* observation presented an expected, female-limited expression pattern, but more intriguingly, a tissue specific expression pattern. Spinach initiate unisexual flowers thus sex determination in spinach occurs during the transition to flowering or during very early in floral development (Pfent, Catherine *et al.*, 2005; Sather, D Noah *et al.*, 2005). *SpFemUn3* was detected in the pre-floral/early floral meristem tissue but not in surrounding vegetative tissues at this early and its specific expression implies that *SpFemUn3* may have some function in female flower identity. We also observed female specific expression in later stages of ovary development, particularly within the nucellus. In most angiosperms the nucellus provides nutrients to the developing megagametophyte and is consumed during this process (Werker, 1997). *Chenopodium quinoa*, a close relative to spinach, is observed to have a nucellus that is not consumed but persists after fertilization, developing into perisperm and functioning as the nutrient resource for the seed (Prego *et al.*, 1998). The quinoa perisperm function is analogous, but not homologous to the heavily studied endosperm function in species of grain (Burrieza *et al.*, 2014). Programmed cell death (PCD) is a required process in the development of both the grain endosperm (Radchuk *et al.*, 2010) and the quinoa perisperm (López-Fernández & Maldonado, 2013). The *in situ* analysis of *SpFemUn3* in combination with our understanding of analogous structures in related species allow one to develop testable hypotheses regarding the function of this spinach specific gene.

Our transcriptome analysis found KQ187820 was overrepresented in male samples and identified as probable pectinesterase/pectinesterase inhibitor 51 via megaBLAST

(Supplemental Table 1). Pectin is a critical element of the cell wall and thus undergoes modification by pectinesterase and related enzymes during plant growth (Ridley *et al.*, 2001). During dehiscence the spinach stamen rapidly extends the filament beneath the anthers causing the anthers to be projected above the rest of the flower. This process requires reorganization of the cell wall and thus male flowers would be expected to display elevated expression of genes such as *SpPect*. However, *SpPect* expression was observed to be elevated in males on average but was not significantly different compared to female expression (Figure 3.1) somewhat contradicting the transcriptome analysis. Using LAMP mediated *in situ* detection of *SpPect* expression, we did not observe significantly different staining between male and female inflorescences (Figure 3.7). In both sexes, *SpPect* signal was strongly detected in young floral tissue which is presumed to be growing quickly thus requiring elevated cell wall modification activity. Spinach male inflorescences are typically larger than a female inflorescence. Were cell mass responsible for the size differences one would expect *SpPect* to be overrepresented in male samples. Unfortunately, it is unclear if this difference in size can be accounted for by cell number or cell mass. Additionally, the discrepancy between the transcriptome and qRT-PCR could be attributed to the methods themselves. The former relies on read count differences while the later normalizes across samples with an internal control, thereby reducing the magnitude of differential expression between sexes. *SpPect* is likely an example of an overexpressed gene that correlates to the morphology of interest but is unlikely to be critical for its development.

Similar to *SpPect* data our observations of *SpFbox* expression using different methods yields conflicting though not unexplainable results. The transcriptome data identified *SpFbox* as overrepresented in male tissue however, qRT-PCR data indicated significant over

expression in female samples. LAMP mediated *in situ* observations of *SpFbox* expression in tissues from both sexes help explain the disagreement. In the early stages of flower development *SpFbox* expression is observed in the reproductive tissues of both sexes (Figure 3.5a, 3.5c). As development continues, expression in the male is reduced eventually being limited to the tapetum with slight expression in the pollen mother cells (Figure 3.5d). In developed females the *SpFbox* expression is reduced in the ovary walls and nucellus but remains strong in the funiculus and integuments (Figure 3.5e). Slight differences in the developmental stage of the tissues used for transcriptome and RT-qPCR analysis could account for the conflicting results. Less mature inflorescences would be expected to overrepresent *SpFbox* expression in male tissue while more developed tissue is expected to have a female bias. The LAMP mediated *in situ* gives insight to the spatial and temporal dynamics of gene expression that neither RNAseq or RT-qPCR can provide.

Our RNAseq analysis produced a preponderance of female reads, nearly 2.5 times more than male reads. The excess female reads likewise produced an excess of female contigs yielding approximately 3.6:1 ratio. Due to this imbalance the differentially expressed transcripts were biased to identify overrepresented male transcripts, indeed only 7 of the 165 transcripts were found to be overrepresented in females. Despite these shortcomings the data produced sensible gene candidates given some biological context. Previous work in our lab has identified the differential expression of *GIBBERELLIC ACID INSENSITIVE* (*SpGAI*) a typically repressive transcription factor that mediates gibberellic acid (GA) hormone response, as critical for unisexual development in spinach (West & Golenberg, 2018). Males were observed to have roughly half the *SpGAI* expression as females, this reduction of *SpGAI* in males suggests that in addition to initiating 'maleness' a GA response would be initiated

in parallel. Crosstalk amongst hormones has long been observed (Weiss & Ori, 2007) and typically, GA acts antagonistically toward abscisic acid and synergistically with auxin. Our differential expression analysis detected enzymes responsible for the degradation of abscisic acid (*Abscisic acid 8`-hydroxylase 1-like*) and synthesis of auxin (*Indole-3-acetic acid-amido synthetase*) were overrepresented in males (Table 1). This observation fits nicely with our understanding of the GA response pathway and male development in spinach. GA is also understood to be involved in stress response (Abbasi *et al.*, 2004; Huerta *et al.*, 2008) which could explain the presence of numerous genes affiliated with stress (Table 1).

Generating large sequence databases will only get more efficient and affordable as technology advances. Researchers can leverage this to investigate key differences between individuals in a population and/or tissues within an organism. Here we present sex specific transcriptomes and identified differentially expressed genes presumably involved in developing the morphological differences observed in spinach flowers. *In situ* analysis of select genes provide much needed context to address discrepancies in the transcriptome and qRT-PCR analysis. Additionally, the *in situ* observations will narrow the focus of future efforts to elicit the function of the genes identified in this study.

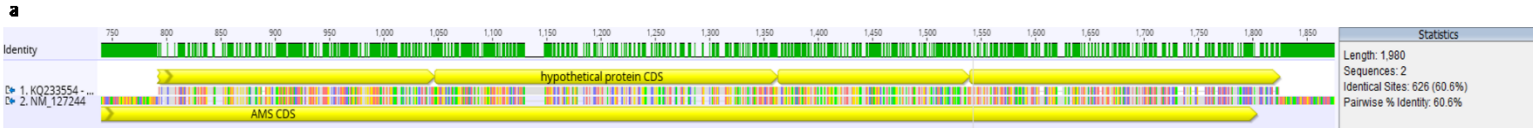
| | |
|---|---|
| SpAbAHydroxqP.318F | TTACCATGCTAAGCTG |
| SpAbAHydroxqP.585R | TTCTCTGTAATTCACCTCG |
| SpAMSqP.86F | CTATCTCAGATAGTGATCCTC |
| SpAMSqP.322R | GGTCTTTTTCTTCCTTCTG |
| SpAPT1qP.3F | CATCTCCATTTTGTCTGTTC |
| SpAPT1qP.192R | TGTTGAAAGATACAAGGGG |
| SpEF1qP.481F | CTTCAGACTCAAAGAATGAC |
| SpEF1qP.693R | CATTCTTCAAGAACTTAGGG |
| SpExpansinqP.65F | GATGTTAAGATTAAGCCGTC |
| SpExpansinqP.268R | GAAGTTTTTGCTGCCTTC |
| SpFboxqP.901F | TCTCTTACCTTATACCTTGG |
| SpFboxqP.1113R | GATGAAGTTTTCCTCTTGAG |
| SpFemunknown1qP.279F | CTACCCCTTCAACTACTATC |
| SpFemunknown1qP.489R | CCAGATTACAAACAAAGTCC |
| SpFemunknown2qP.50F | ATAGGAACCTGATTTCTGG |
| SpFemunknown2qP.257R | CCACAAATCAATAGACTCAG |
| SpFemunknown3qP.281F | TGGTCATAAGTTCTGGAC |
| SpFemunknown3qP.490R | AGAGGTACTAGATGAGGC |
| SpFemunknown4qP.1F | GTATTTATAGGCGATTCTTGCG |
| SpFemunknown4qP.204R | CCTAATTGCTATACCCAGGTAC |
| SpGAUTqP.31F | GTCTTCTAACCATGTTCTTG |
| SpGAUTqP.241R | AGCTGTTGATCATAGATGG |
| SpHS70_3alphaqP. 2958F | GGGTGAAGATAAAGAGTTTG |
| SpHS70_3alphaqP.3164R | GTAGGCTCGTTAATAATTCG |
| SpIAASynqP .4668F | CTAGAGTACACTAGCTACG |
| SpIAASynqP.4878R | CATTCTCCACTACCTTAATC |
| SpNACLqP.221F | TTAGTCCAAGAGATAGGAAG |
| SpNACLqP.425R | CGATACTCATGCATAATCC |
| SpPectinestqP.3595F | GCAGGAATTAGTTTGCTC |
| SpPectinestqP.3804R | CAAGCACAAGAACTACTTAG |
| SpUBQ5qP.5F | CAGATTTTCGTGAAAACCC |
| SpUBQ5qP.208R | TGAAGAGTTGATTCCTTCTG |

***Supplemental Table 3.1*** *– List of primers used for qPCR survey. The number following the gene name indicates primer position relative to the start codon and sequence listed in 5` to 3` orientation.*

| | |
|---|---|
| SpAMS.782F3 | GGGACTCGTTGCTAGAGG |
| SpAMS.959B3 | AGTGGTTGTGGAAAGGAC |
| SpAMS.800FIP | TCCACTTCCATTTTCTGATGATGCTCAACACGTAATCAAGCAGGA |

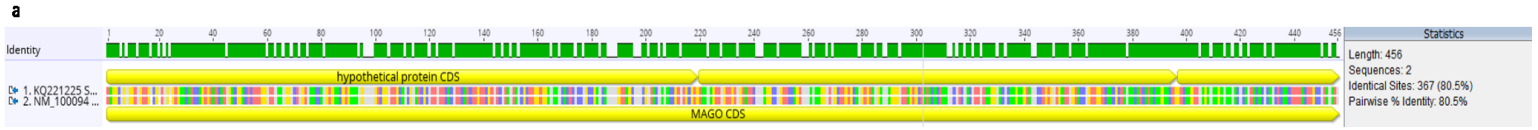
| | |
|---|---|
| SpAMS.936BIP | CGACAGCAGAATCAGAATCAGAATCCTATATGATGGTTGTGAATGTGT |
| SpFBox.192F3 | GCCTGATGCTATCCTTCA |
| SpFBox.399B3 | AGACTTGTTGATGATGAGGA |
| SpFBox218.FIP | TCCCTCTCCATCGTTTGGAAATTTCTCTCTAATCCCAATAAAATCCG |
| SpRBox.343BIP | CTGCTGATCATCTTGAATATGCCAAGAAAACTCATTTGGTGTCTG |
| SpFBox.315LB | AGACTTTGGAGAAGAGTTTGCCAAA |
| SpFemUn3.173 F3 | GCACTCTGGGTCAGTACGAT |
| SpFemUn3.369 B3 | AGTAGGGACCCCAAACTGTA |
| SpFemUn3.250 FIP | GGAACACGGGAGGTTCTGGAAACTCATCTTCCTCCGAGGGA |
| SpFemUn3.286 BIP | CCTTAGCGGCTAGGCCGTTCTTGGGCATGACACCAAAGA |
| SpFemUn3.213 LF | TCTTCTACAAGAAGAACAAATCCCC |
| SpFemUn3.307 LB | GTGCCCTTTTCAAGCTCTTACA |
| Mago Nashi.340 F3 | TGAAGGAAGATGATAACCTCTG |
| Mago Nashi.531 B3 | AGTGAAGTGAGATAAGTGAGAA |
| Mago Nashi.426 FIP | ATGAGATACGCTCATTGCCCAGAACCTGATGTTATTGGGAGG |
| Mago Nashi.446 BIP | TTCCCTTGCTGATGTCCAGAAACACTTCAAGTCCTGAACAA |
| Mago Nashi.446 LB | GTAGTAAAGATCCCGAAGGACTTC |
| SpPectinase.283 F3 | GGTCAGTCTATGGTCCAGCA |
| SpPectinase.496 B3 | TGGAGGCCGAATCATTCAC |
| SpPectinase.362 FIP | TTCCCCAGAACCTCAAGGCACCCGACTCTGCCCATAATCCT |
| SpPectinase.414 BIP | ACGTGGGAAGCTAAAGGACGCAGACCTGACCAGCAGTCAT |
| SpPectinase.435 LB | ACGTGCATGGACTAGCGC |

***Supplemental Table 3.3*** *– List of primers used for LAMP mediated in situ hybridization. The number following the gene name indicates primer position relative to the start codon and sequence listed in 5` to 3` orientation.*




***Supplemental Figure 3.1*** *– Sequence alignment of* SpAMS *and* AtAMS. *The spinach sequence (KQ233554) on top with the* A. thaliana *sequence (NM_127244) below. The green bar along the top indicates matching bases, the alignment shows 60% sequence similarity.*






***Supplemental Figure 3.2*** *– Sequence alignment of* Fbox . *The spinach sequence (KQ203887) appears on top in both (a) and (b) panels, the green bar along the top of both panels indicate matching base pairs. (a) Spinach* Fbox *aligned to* A. thaliana *(NM_001161177) with 50.3% sequence identity. (b) Spinach* Fbox *aligned to* Beta vulgaris *(XM_0192472) with 81.9% sequence similarity.*


***Supplemental Figure 3.3*** – *Sequence alignment of* SpMago *and* AtMago. *The spinach sequence (KQ221225) on top with the* A. thaliana *sequence (NM_100094) below. The green bar along the top indicates matching bases, the alignment shows 80.5% sequence similarity.*

# CHAPTER 4: DIRECT INTERACTION BETWEEN *SPINACIA OLERACEA* L. *LEAFY* AND *GIBBERELLIC ACID INSENSITIVE* TRANSCRIPTION FACTORS OBSERVED *IN VIVO* AND *IN PLANTA*

## INTRODUCTION

Although the majority of angiosperms are hermaphroditic, a small but significant number of species segregate the staminate and pistillate flowers between individuals and are described as dioecious. The multiple evolutionary origins for this reproductive strategy exclude a unifying theory to explain the mechanism by which dioecious plants accomplish sexual segregation. In *Spinacia oleracea*, cultivated spinach, we have proposed a mechanism in which expression of *GIBBERELLIC ACID INSENSITIVE* (*SpGAI*) is critical for feminization while inhibition of *SpGAI* is observed to masculinize the individual (West & Golenberg, 2018). Previous studies demonstrated that the B class floral organ identity genes, *SpPI* and *SpAP3*, are expressed only in males and act to trigger stamen development in third whorl primordia as well as to suppress fourth whorl organ initiation (Pfent, Catherine *et al.*, 2005; Sather *et al.*, 2010). The functional analysis of SpGAI through inhibition of proteasome degradation or decreased gibberellic acid (GA) versus *SpGAI* expression or increased GA indicates that SpGAI feminizes spinach floral development through epistatic suppression of *SpPI* and *SpAP3* (West & Golenberg, 2018). In *Arabidopsis thaliana,* the inflorescence identity gene *LEAFY* (*LFY*) is a direct activator of B class floral organ identity gene activity (Weigel *et al.*, 1992). To unite these observations, we hypothesize that a direct interaction between *SpGAI* and *LEAFY* (*SpLFY*) may regulate alternative unisexual development in spinach. However, it is unknown if these transcription factors interact physically or through indirect means.

*SpGAI* is a member of the GRAS domain transcription factor family and possesses a DELLA motif, a conserved 17 amino acid sequence near the N-terminus. DELLA transcription factors have been observed as the main elements affecting a response to the phytohormone gibberellic acid (GA). Typically, DELLA transcription factors inhibit expression of target genes and upon reception of the GA signal are polyubiquitinated at the DELLA motif and subsequently degraded via the 26S proteasome (Spartz, Angela K & Gray, William M, 2008; Sun, 2010). In contrast to the five DELLA proteins in *Arabidopsis thaliana* (Lee *et al.*, 2002) only one is found in spinach. Thus, we presume SpGAI to be the main transcription factor involved in GA response (West & Golenberg, 2018). In *A. thaliana* DELLA proteins have been observed to interact physically with *CONSTANS* (CO), a critical member of the photoperiod pathway of flowering (Xu *et al.*, 2016) and *FLOWERING LOCUS C* (FLC), a critical member of the vernalization pathway of flowering (Li *et al.*, 2016) both of which are important transcription factors upstream of *LEAFY* (*LFY*). Interestingly, a portion of the cis regulatory region preceding *LFY* was found to be required for GA mediated flowering under short day conditions (Blazquez & Weigel, 2000) and this segment was found to interact with AtMYB33 in a GA dependent manner (Gocal *et al.*, 2001), but we were unable to find reports of DELLAs directly interacting with MYB33 or LFY.

*LFY* expression is observed to be a key factor in the transition from vegetative growth to flower development (Schultz & Haughn, 1991). *LFY* expression is initiated by *SUPPORESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), a transcription factor that integrates flowering signals from numerous pathways including the photoperiodic and vernalization pathways (Lee *et al.*, 2000; Onouchi *et al.*, 2000; Yoo *et al.*, 2005). Once expressed, *LFY* interacts with floral organ identity genes *APETALA 1* (*AP1*) (Mandel &

Yanofsky, 1995; Wagner *et al.*, 1999), *AGAMOUS* (*AG*) (Huala & Sussex, 1992), and *APETALA 3* (*AP3*) (Weigel & Meyerowitz, 1993) with the help of *UNUSUAL FLOWER ORGANS* (*UFO*) (Lee *et al.*, 1997) all of which interact to define the organs of the flower (Coen & Meyerowitz, 1991; Pelaz *et al.*, 2000).

Spinach develops unisexual flowers from inception and does not have a transient hermaphroditic phase suggesting that the sex of the flower is determined at some point during the initiation of the flower. Previous work in our lab has found that expression of *SpGAI* to be critical for the development of female flowers and when *SpGAI* expression is reduced in females with VIGS treatment male flowers begin developing upon an otherwise female individual (West & Golenberg, 2018). Our model suggests that in female spinach *SpLFY* promotes the expression of the B and C class genes as expected from *A. thaliana* models however, the presence of *SpGAI* inhibits the expression of only the B-class genes thus preventing the development of male organs but not other flower organs. In male spinach *SpGAI* expression is lower than in females which may allow *SpLFY* to interact with its canonical targets. The combinatorial expression of B- and C-class genes initiate the androecium as would be expected however, the spinach B-class genes are observed to have a novel function that inhibit the development of the gynoecium (Sather *et al.*, 2010). To better understand how the spinach B-class genes are selectively initiated we need to explore how *SpGAI* prevents *SpLFY* from initiating B-class expression in females. *SpGAI* may bind *SpLFY* directly preventing its interaction with B-class gene promoters. We begin exploring possible direct interactions between *SpGAI* and *SpLFY* utilizing an *in vivo* yeast two-hybrid screen and *in planta* with a bimolecular florescence complementation screen.

## METHODS

### Generation of mutant *SpLFYp-q* and *SpGAIΔDELLA*

Site-directed mutagenic PCR was used to generate a base pair substitution in SpLFY that would result in a replacement of the proline residue at position 369 in the polypeptide with a glutamine residue and replace the stop codon with a valine residue. Mutagenic primers were designed with an 18bp overlap that included the desired base substitution. Two separate PCR reactions were performed designed to amplify SpLFY from the start codon to the mutation site in one reaction using LFY F and LFYp369q Rev primer pairs, and from the mutagenic site to the stop codon in the second reaction using LFYp369q F and LFY NSC Rev primer pairs, all primer sequences listed in Table S1. The reactions were cleaned using Wizard SV PCR clean up kit (Promega) and ran on 1.5% agarose gel to confirm amplification of desired product. The cleaned products were combined and used as template for a reaction to anneal the separate halves and amplify the full length, mutated SpLFY amplicon using LFY F and LFY NSC Rev primers. The product size was checked on a 1.5% agarose gel, cleaned using the Wizard SV PCR clean up kit, and cloned into pDONR/Zeo with a BP clonase reaction following manufacturer's instructions (Invitrogen). The reaction product was transformed into DB3.1 competent cells following standard heatshock protocol. Successful transformation was selected for on 1.5% LB agar plates with zeocin (30µg/mL). A PCR screen using gene specific primers confirmed positive transformation and colonies harboring an insert of the proper size were cultivated and used to produce purified plasmid, sanger sequencing was used to check the fidelity of our site directed SpLFYp369q mutation.

A similar approach was used to generate a mutated *SpGAI* encoding clone. The construct was designed to delete the region encoding for the 17 amino acid DELLA motif (DELLAVLGYKVRSSDMA). Sequence information for the following primers can be found in Table S1. A fragment from nine bases before the start codon to position 123 in the coding sequence was amplified. A second fragment was generated from position 178 to the end of the gene. The forward primer for the second fragment included a 13 nucleotide 5' linker that complemented the 3' end of the first fragment. The reverse primer mutated the stop codon. The fragments were cleaned as above and used together as templates to create a single fragment using forward and reverse primers with BP clonase extensions. Cloning into pDONOR/zeo was performed as described above and the sequence of the clone was verified by sequencing.

**Cloning into Yeast2Hybrid and BiMolecular Florescent Complementation Vectors**

Genes of interest were initially cloned into pDONR/Zeo vectors. PCR amplification was used to attach attB recognition sequences to the 5` and 3` ends of our genes of interested with primers listed in Table S1**.** The PCR product was cleaned with Wizard SV Gel and PCR clean up kit (Promega) and shuttled into the pDONR/Zeo vector facilitated by a Gateway BP reaction according to the manufacturers protocol (Invitrogen). These constructs were used to transform *E. coli* via standard heat shock method and colonies were screened for positives on 1.5% (g/v) LB agar with zeocin (30µg/mL). The inserts were then transferred from pDONR/Zeo versions into pNLexAttR, pJZ4attR, pDEST-VYCE, and pDEST-VYNE vectors with the Gateway LR reaction, and the plasmid constructs transformed into *E. coli* and selected on LB agar plates with appropriate antibiotics.

**Yeast2Hybrid Screen**

pNLexAattR vectors with the genes of interest were used to transform yeast strain RFY306 (MATa, *his3A200, leu2-3, lys2A201, ura3-52, trplA::hisG*) (Finley & Brent, 1994). The transformed yeast will express genes of interest fused to the LexA DNA binding domain. pJZ4attR vector species were transformed into RFY231 (MATα, *trp1Δ::hisG, his3, ura3-1, leu2::3*Lexop-*LEU2*) which will produce our genes of interest fused to the B42AD transcription activation domain (Kolonin & Finley, 1998). All yeast were transformed following the standard LiOAc method, positive transformants were selected by plating on SD -ura -his (for pNLexAattR) or SD -ura -trp. We performed yeast two-interaction assay through mating the alternatively transformed strains following the protocol described previously (Kolonin *et al.*, 2000). Positive protein interactions were identified by growth on leucine drop-out media.

**BiMolecular Florescent Complementation in Onion**

*Agrobacterium tumefaciens* strain GV3101 with C58C1/pMP90 background was transformed via heatshock with BiFC vectors p DEST-VYCE and p DEST-VYNE (Waadt *et al.*, 2008) harboring genes of interest and selected on YEB agar plates containing gentamycin (20µg/mL), rifampicin (50µg/mL), and kanamycin (30µg/mL). Transformed *A. tumefaciens* was used for transient expression of gene constructs in onion epidermal cells as previously described by Sun *et al.* (2007). In brief, transformed *A. tumefaciens* was grown to saturation in YEB medium then an aliquot was diluted 1/10 into media containing 5% (g/v) sucrose, 100mg/L acetosyringone and 0.01% (v/v) Silwet-77 and incubated with onion scales for 12-24hrs at 28°C with gentle agitation. The onion scale was then transferred to ½ Murashige

and Skoog 0.7% (g/v) agar plates and incubated at 28°C for 24-48hrs. For observation, onion scales were washed in deionized water, epidermal layer was gently peeled and transferred to a glass slide. The epidermis was then observed under a uV microscope using Leica model DM5500B.

## RESULTS

### Yeast Two-Hybrid Analysis

We begin exploring the possible direct interaction of SpLFY and SpGAI with a yeast two-hybrid (Y2H) screen. A major benefit of the method is the ability to test numerous potential interactions in parallel. In addition to SpLFY and SpGAI we were interested in testing for any unexpected interactions between SpUFO, SpAP3, and SpPI as well as the mutants we constructed SpLFYp-q and SpGAIΔDELLA. The SpLFYp-q mutant substitutes the amino acid proline for a glutamine at the 369$^{th}$ position in the polypeptide. This region is predicted to function in protein-DNA interactions and by replacing a nonpolar residue with a polar amino acid we hope to disrupt this activity. The SpGAIΔDELLA mutant had the conserved DELLA motif removed from its N-terminal end, this motif is understood to be polyubiquitinated which tags the transcription factor for degradation (Dill *et al.*, 2001). The expression of all genes of interest is driven by a GAL4 promoter. As such, YPD dropout plates with glucose as a carbon source should not activate gene expression and any colonies on glucose containing plates represent false positive interactions. Unfortunately, the LexA DNA binding domain fused with SpPI and SpUFO produced robust colonies when plated on glucose, while the SpGAIΔDELLA and SpLFY LexA fusions produced sparse colonies (Supplemental Figure 1). No autoactivation was observed in any of the genes fused to the

B42AD transcription activating domain on the glucose plates including SpPI and SpUFO (Supplemental Figure 1).

When plated on YPD dropout plates with galactose, gene expression is activated and physical interaction between genes of interest is expected to result in growth. Due to the autoactivation observed on glucose plates the colonies produced via interaction with the LexA fused SpPI and SpUFO series are ignored. The LexA fused SpGAIΔDELLA grown on galactose plates produced sparse colonies mimicking growth observed on the glucose plates indicating no interaction with any genes of interest (Supplemental Figure 1). SpLFY fused with the LexA DNA binding domain produced evidence of multiple interactions however, when fused with the B42AD transcriptional activation domain no reciprocal interactions were observed (Figure 4.1). SpLFY physically interacts with SpGAI and SpGAIΔDELLA indicating protein-protein binding does not require an intact DELLA motif. SpLFY also interacts with SpUFO which is in agreement with previous observations in *A. thaliana* (Levin & Meyerowitz, 1995). Physical interaction between SpPI and SpLFY is also observed. Interestingly, SpLFY was not observed to form a dimer with SpLFY as reported elsewhere (Siriwardana & Lamb, 2012) or to interact with the mutant SpLFYp-q. All of the interactions observed involving SpLFY were not seen in the SpLFYp-q mutant suggesting our engineered mutation successfully altered wild type function. In contrast, none of the LexA fused SpGAI combinations produced evidence of interaction.

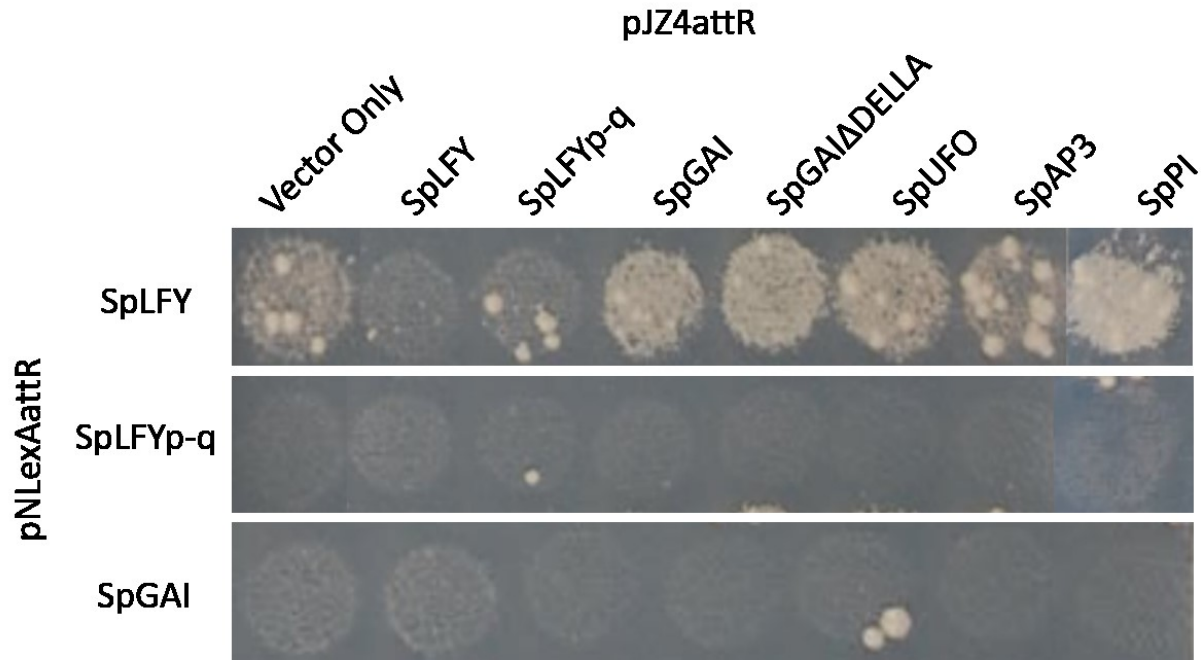



***Figure 4.1*** *– Yeast two-hybrid screen, galactose plate. pNLeXAattR species contain the LexA DNA binding domain upstream of the gene of interest. pJZ4attR species contain B42AD activation domain upstream of the genes listed. Matings were plated on YPD –leu –his –ura –trp with galactose, growth indicates successful interaction between indicated proteins.*

**BiMolecular Fluorescence Complementation**

The yeast two-hybrid assay is an excellent and high throughput method for screening protein interactions, but it is not without drawbacks. Notably relevant to our study is the possibility for autoactivation when transcription factors are being screened as well as the lack of rescue/signal in reciprocal combinations. Although these shortcomings can explain the results observed in the yeast two-hybrid analysis it certainly casts doubt on the interactions characterized. In order to confirm the observations made *in vivo* with the yeast two-hybrid screen, we chose an *in planta* Bimolecular Florescence Complementation (BiFC) analysis. This method is beneficial as the transcription factors in question will be challenged

in an environment more closely resembling wild type circumstances in not only intracellular conditions but also the potential presence of orthologous versions of hitherto unknown co-factors. We began by co-infiltrating onion with pDEST-VYCE:SpAP3nmb and pDEST-VYNE:SpAP3nmb serving as our negative control. The brightfield DIC image shows healthy onion cells with a nucleus clearly visible (Figure 4.2a) while the fluorescent darkfield image shows no indication of significant signal (Figure 4.2b) and no interaction is observed in the overlay image (Figure 4.2c). To serve as a positive control we used pDEST-VYCE:SpAP3nmb and pDEST-VYNE:SpPInmb as they are understood to form a heterodimer (Riechmann et al 1996). Similar to the negative control, although at a higher magnification, healthy onion cells with clearly visible nuclei are observed (Figure 4.2d). However, in the fluorescent darkfield we observe strong and localized signal (Figure 4.2e) and when overlaid with the brightfield image the fluorescent signal is being produced in the nuclear region of the cell (Figure 4.2f).

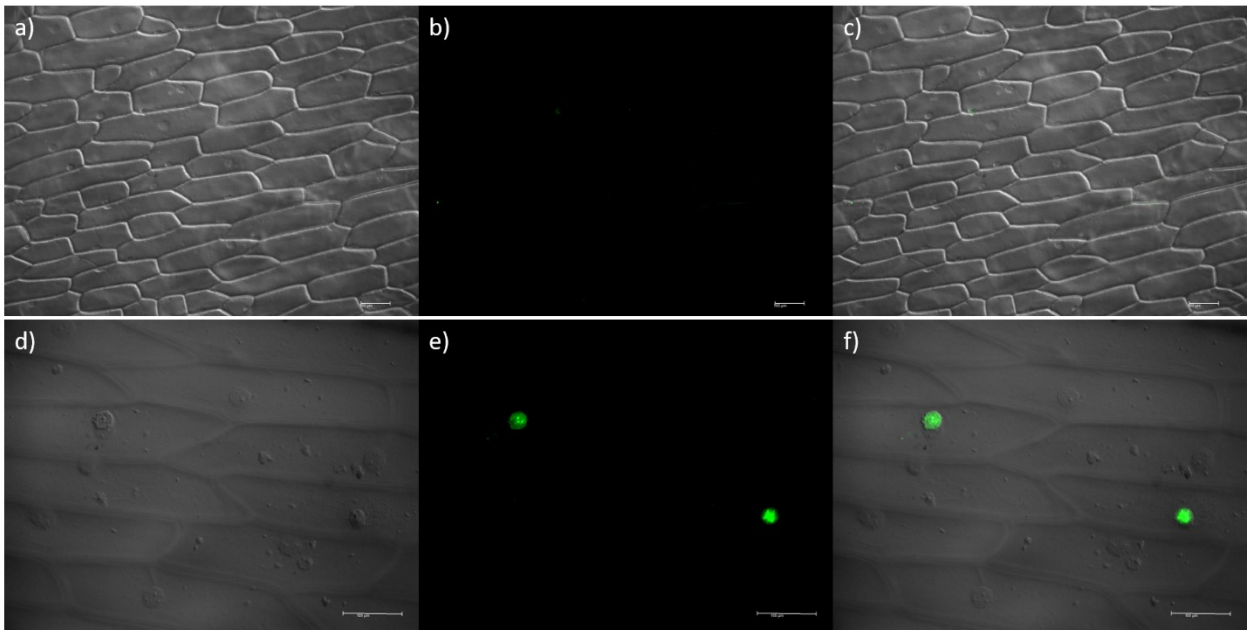



***Figure 4.2*** *– In Planta Bimolecular Florescence Complementation Controls. (a-c) pDEST-VYCE:SpAP3nmb pDEST-VYNE:SpAP3nmb co-transfection series. a) Brightfield DIC of onion tissue. b) Florescence darkfield with green false color. c) Overlay of a and b. d-f) pDEST-VYCE:SpAP3nmb pDEST-*

*VYNE:SpPInmb co-transfection series. d) Brightfield DIC. e) Florescence with false color. f) Overlay of d and e. Scale bar is 100µm.*

In order to test for direct interaction of SpLFY and SpGAI and strengthen our observations from the yeast two-hybrid screen we co-infiltrated onion epidermal tissue with SpLFY and SpGAI. In agreement with the yeast two-hybrid assay we observed fluorescent signal when onion epidermal tissue was co-infiltrated with pDEST-VYCE:SpLFY and pDEST-VYNE:SpGAI (Figure 4.3b) and this signal was confined to the nucleus of the cells (Figure 4.3c). This indicates a direct interaction between the two transcription factors and corroborates our yeast two-hybrid findings. Previous work had identified *UFO* as being important for *LFY*s activation of B-class genes (Lee *et al.*, 1997). To test if this interaction is direct and conserved in spinach we co-infiltrated onion scales with pDEST-VYCE:SpLFY and pDEST-VYNE:SpUFO. We observed discrete fluorescent signal indicating a physical interaction between SpLFY and SpUFO (Figure 4.3e) and this signal was localized to the nucleus (Figure 4.3f).

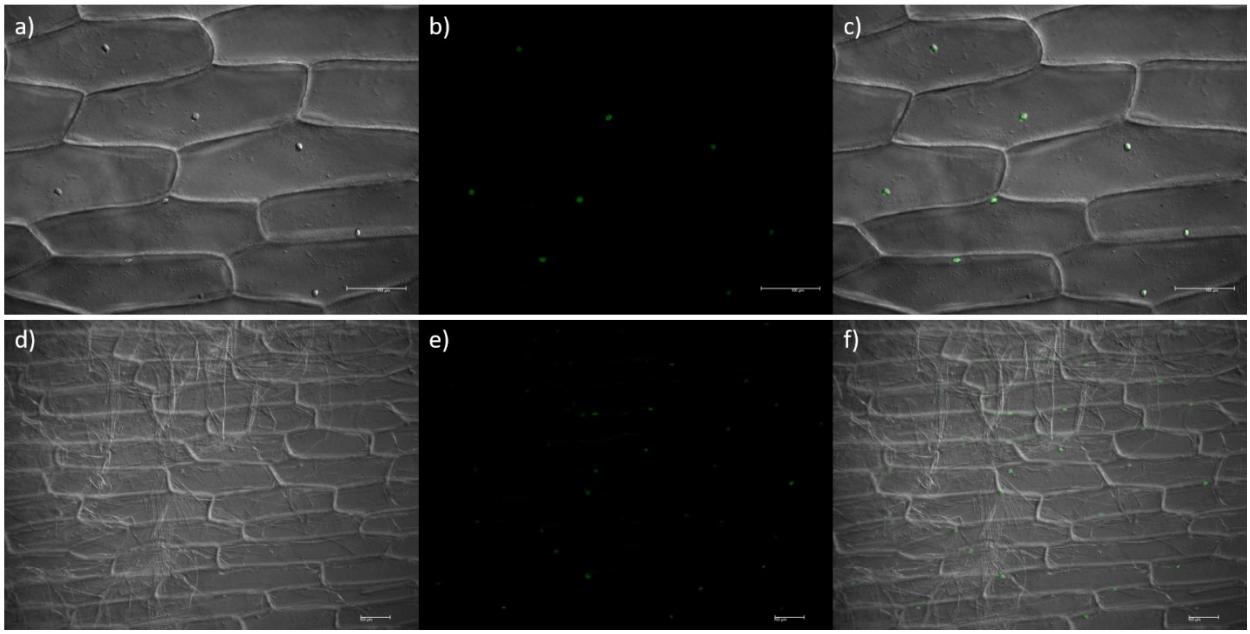
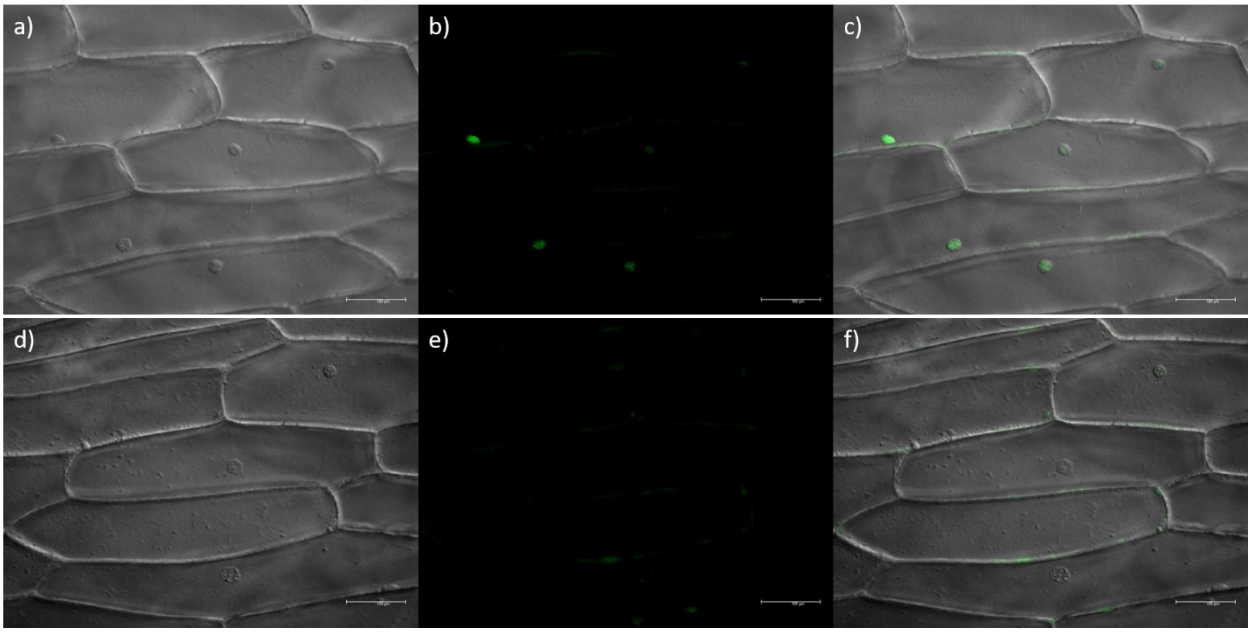


***Figure 4.3*** *– In planta co-transfection of SpLFY with SpGAI and SpLFY with SpUFO. a-c) pDEST-VYCE:SpLFY pDEST-VYNE:SpGAI co-transfection of onion tissue. a) Brightfield DIC. b) Darkfield florescence with green false color. c) Overlay of a and b. d-f) pDEST-VYCE:SpLFY pDEST-VYNE:SpUFO co-transfection. d) Brightfield DIC. e) Darkfield florescence with green false color. f) Overlay of d and e. Scale bar is 100µm.*

Our model suggests SpGAI prevent the activation of B-class genes and we have observed that SpLFY and SpGAI interact physically as well as a direct interaction of SpLFY and SpUFO. To explore a possible interaction between SpUFO and SpGAI we co-infiltrated onion scales with pDEST-VYCE:SpGAI and pDEST-VYNE:SpUFO. Strong signal was observed in the fluorescent darkfield imaging (Figure 4.4b) and when overlaid with the brightfield image the signal is localized to the nucleus (Figure 4.4c). The ability of SpLFY, SpGAI, and SpUFO to interact with one another opens the possibility for heteromultimeric complexes. Previous work has shown that in *A. thaliana* LFY is understood to form homodimers that are essential for proper function (Siriwardana & Lamb, 2012). To test for potential homodimerization of SpGAI we co-infiltrated onion with pDEST-VYCE:SpGAI and pDEST-VYNE:SpGAI. Although the onion cells appear healthy with nuclei clearly visible (Figure 4.4d) no fluorescent signal above background levels were observed (Figure 4.4e-f). Thus, it does not appear that SpGAI is able to form homodimers.

***Figure 4.4*** *– In planta co-transfection of SpGAI and SpUFO as well as SpGAI and SpGAI (Figure on next page). a-c) pDEST-VYCE:SpGAI pDEST-VYNE:SpUFO co-transfection of onion tissue. a) Brightfield DIC. b) Darkfield florescence with green false color. c) Overlay of a and b. d-f) pDEST-VYCE:SpGAI pDEST-*

*VYNE:SpGAI co-transfection. d) Brightfield DIC. e) Darkfield florescence with green false color. f) Overlay of d and e. Scale bar is 100µm.*




## DISCUSSION

Spinach plants are unisexual from floral initiation and we have found the transcription factor *SpGAI* is critical for alternate sex-determination. Elevated expression of *SpGAI* in females inhibits activation of the B-class genes. When female *SpGAI* expression is transiently knocked-down, male flowers begin to develop (West & Golenberg, 2018). Expression of the B, C, and E class floral organ identity genes is understood to be activated by LFY (Irish, 2010) which opens the possibility that SpGAI interacts with SpLFY and prevents the activation of B-class genes in spinach. To explore this possibility, we utilize a yeast two-hybrid (Y2H) screen and bimolecular fluorescence complementation assay (BiFC) to detect physical interaction between the transcription factors of interest.

Our observations from the Y2H screen indicates direct interaction between SpLFY and SpGAI, SpGAIΔDELLA, and SpUFO (Figure 4.1). To our knowledge this is the first

observation of direct interaction between orthologs of LFY and DELLA family transcription factors. The interaction between SpLFY and SpGAI does not require the presence of the characteristic DELLA motif as evidenced by the SpLFY – SpGAIΔDELLA Y2H combination. The DNA binding domain of LFY was identified via deletion analysis to stretch from amino acid 320 to 507 within the highly conserved C domain (Maizel *et al.*, 2005). To study the potential effect of DNA binding on function we designed a mutant replacing a proline at the 369$^{th}$ position with glutamine. Interestingly, all SpLFY interactions were abolished in the SpLFYp-q mutant suggesting that the single amino acid replacement was enough to abolish protein interactions. To confirm our initial observations many of the interaction assays were repeated *in planta* utilizing a BiFC screen. We observed SpLFY interacting with SpGAI and SpUFO (Figure 4.3) corroborating data from the Y2H screen. Additionally, we observe SpGAI to interact with SpUFO *in planta* but not in the yeast two-hybrid assays suggesting that plant specific factors may be required to facilitate this interaction. From these combined data it appears that SpGAI interacts directly with SpLFY and SpUFO.

LFY is integral for proper flowering as its expression is the culmination of pro-flowering signals from multiple pathways responsible for determining how permissive the external and internal environments are to reproduction. Briefly, the main pro-flowering signals are represented by *CONSTANS* (*CO*) which activates expression of *FLOWERING LOCUS T* (*FT*) which in turn activates *SUPPORESSOR OF CONSTANS OVEREXPRESSION 1* (*SOC1*), the generally repressive of *FLOWERING LOCUS C* (*FLC*) can inhibit the expression of both *FT* and *SOC1*. Once expressed, *SOC1* then activates *LFY* expression and a commitment to flowering has been made. LFY will then initiate the expression of floral organ identity genes (Coen & Meyerowitz, 1991; Pelaz *et al.*, 2000). DELLA transcription regulators have been found able

to repress the activation of *LFY* through inhibition of the pathway at various points. Recently, evidence has shown that DELLAs is able to bind CO and FLC directly. Binding of CO sequesters and prevents CO from activating downstream targets like FT (Xu *et al.*, 2016). When interacting with FLC, the DELLA-FLC complex has enhanced repression of SOC1 and FT (Li *et al.*, 2016). Additionally, DELLAs have been known to inhibit FT and SOC1 through indirect pathways for approximately a decade (De Lucas *et al.*, 2008; Wang *et al.*, 2009; Wu *et al.*, 2009; Kim *et al.*, 2012; Kumar *et al.*, 2012; Yu *et al.*, 2012). Thus, DELLAs can modify a plant's ability to flower through multiple pathways by suppressing activators of pro-flower signaling and suppressing the signal integrators themselves. DELLAs are also able to influence floral development after a commitment to flowering has been made. The AP1 transcription factor that act synergistically and downstream of LFY has been observed to initiate expression of *GA3ox1*, which encodes an enzyme controlling the rate-limiting step in GA production, as well as the *RGA-LIKE2* a member of the *A. thaliana* DELLA family (Kaufmann *et al.*, 2010). The interaction between SpLFY and SpGAI must be confirmed in other organisms and its functional relevance put into the context of transition to flowering and development of floral organs.

Consistent evidence for interaction between SpLFY and SpGAI both *in vivo* and *in planta* is consistent with our model for alternative sex determination in spinach through epistatic B-class repression by SpGAI. These data suggest that SpGAI is able to bind SpLFY, likely at the DNA binding domain of SpLFY. It is still unclear how the physical interaction between SpGAI and SpLFY prevents the initiation of *SpAP3* and *SpPI*. SpLFY possesses DNA and protein binding domains (Parcy *et al.*, 1998; Hamès *et al.*, 2008). Our Y2H screen indicated the DNA binding domain of SpLFY is required for interaction with SpGAI however,

our assays did not address the protein binding domain either separately or in combination with the DNA binding domain. DELLA transcription regulators are understood to bind transcription promoters preventing them from binding the cis-regulatory regions of target genes (De Lucas *et al.*, 2008; Feng *et al.*, 2008; Xu *et al.*, 2016). It is therefore possible that SpGAI binds to the DNA binding domain of SpLFY and sequesters it in such a manner to prevent SpLFY from activating transcription. How this interaction prevents SpLFY from specifically initiating B-class, but not C-class expression is unclear and going forward the potential influence of B-class cis-regulatory DNA regions must be addressed.

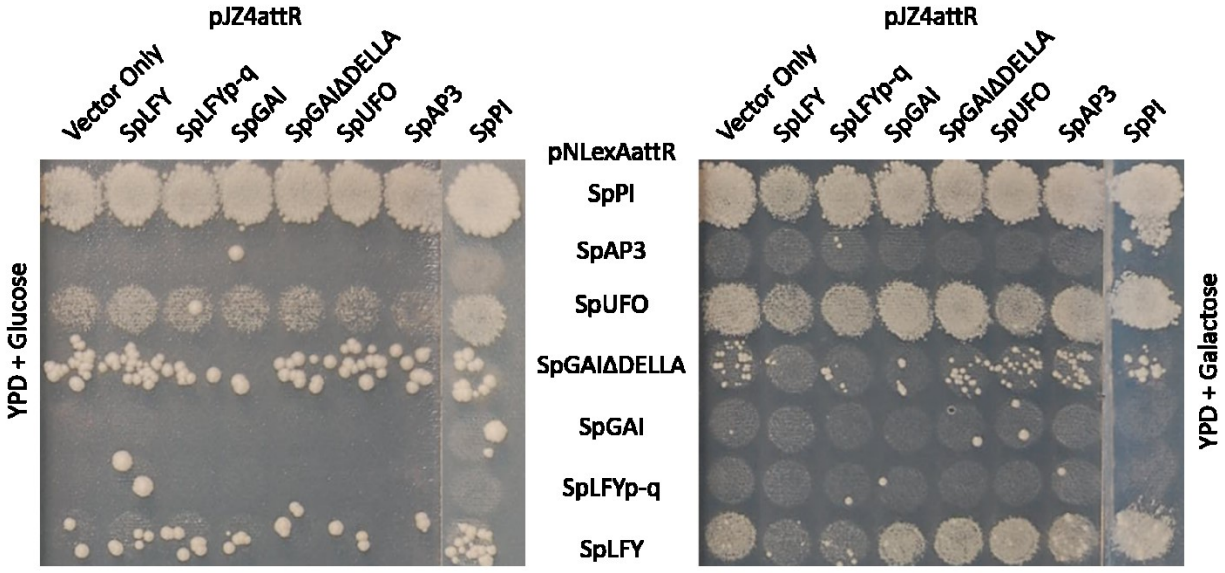
It has been previously established that initiation of B-class transcription requires LFY and UFO activity (Lee *et al.*, 1997). Our consistent observation between the *in vivo* and *in planta* assays indicate that in spinach LFY and UFO can physically interact which agrees with previous observations in *A. thaliana* (Levin & Meyerowitz, 1995). The data indicating that SpGAI and SpUFO physically interact was unexpected and only observed *in planta*. UFO is an F-box protein and likely interacts with the Skp-cullin-Fbox (SCF) E3 ligase complex (Hershko & Ciechanover, 1998; Samach *et al.*, 1999). SCF is responsible for the poly-ubiquitination of protein targets which are then degraded by the 26S proteasome and this pathway of degradation is utilized to remove DELLA transcription factors (Fu *et al.*, 2002; McGinnis *et al.*, 2003; Dill *et al.*, 2004). Given that SpGAI, SpLFY and SpUFO all interact with each other it is tempting to suggest that SpGAI prevents SpLFY from activating the expression of B-class genes and this repression is lifted with the activity of SpUFO. Although multiple allelic versions of SpUFO were identified in *S. oleracea* the alleles were not strongly correlated with sex (Naeger & Golenberg, 2016). Additionally, 694 potential F-box genes have been identified in *A. thaliana* (Gagne *et al.*, 2002). Alternatively, SpGAI could bind to and sequester

SpUFO preventing it from interacting with SpLFY, thereby prohibiting activation of the B-class genes. This repression could be lifted through the degradation of SpGAI via SCF-E3 ligase with a thus far unidentified F-box intermediary. Additional experimentation is required to distinguish between these possibilities.

Determining the physical interactions between transcription factors of renowned importance in the process of flower determination and development will help the field as we move forward in characterizing the many complex interactions required for proper floral development. The physical interactions observed between SpLFY, SpGAI, and SpUFO help refine our mechanism of sexual determination in spinach but the next step will be to observe these interactions in the context of DNA from the regulatory regions of the B-class genes. DELLA transcription factors have long been known to influence the process of flowering but typically though indirect mechanisms (De Lucas *et al.*, 2008; Yu *et al.*, 2012). Our data indicating a direct interaction between SpLFY, the gene critical for integrating the signal to flower and initiating expression of organ identity genes, and SpGAI a member of the DELLA family of transcription factors, is the first of its kind. These direct interactions provide another method of regulation for the critical process of flower development.

| | |
|---|---|
| SpLFY P369Q F | ACATCAACAAACAGAAAATGAGGCACTAT |
| SpLFY P369Q R | TTTCTGTTTGTTGATGTATGTTGCTCCTG |
| SpGAI.-9F | AGAAAAACAATGAAGAGGGAGCTA |
| SpGAI.123R | CATCCCCCCGTCGTTTTG |
| SpGAI.112delta178 | CGACGGGGGGATGGAAGTCGCACAAAAGCTAGAAC |
| GAI NSC Rev | ATCCGTGACGAATTGGCG |
| BP AP3 NMB F | GCAGGCTTAATGCGGAACCACCACGTGTG |
| BP SpAP3 NSC Rev | CAAGAAAGCTGGGTCAACCACATGATCCTGCACCAGC |
| BP PI NMB F | GCAGGCTTAATGAGCCCCTCTACTCCG |
| BP SpPI NSC Rev | CAAGAAAGCTGGGTCAACCACCCTTCCCTGTTGG |
| BPSpGAIF | ACAAAAAAGCAGGCTTAATGAAGAGGGAGCTACCC |
| BP SpGAI NSC Rev | CAAGAAAGCTGGGTCAACGTGACGAATTGGCGATTTGC |
| BP LFY F | GCAGGCTTAATGGATCAAGACCCGTTTAC |
| BP SpLFY NSC Rev | CAAGAAAGCTGGGTCAACGAAAGGAAGATGGTGGG |
| BpUFO F | GCAGGCTTAATGGAAACTTTCAATGTTATC |
| BP SpUFO NSC Rev | CAAGAAAGCTGGGTCAACACCACGAAAGGATCACC |
| BPext F uni | GGGGACAAGTTTGTACAAAAAAGCAGGC |
| BPext NSC Rev uni | GGGGACCACTTTGTACAAGAAAGCTGG |

***Supplemental Table 4.1*** *– List of primers used to generate* SpLFYp369q *and* SpΔDELLA *mutants and Gateway clones. All BP primer pairs designed to attach BP clonase recognition sites 5` of the start codon and 3` of the mutated stop codon, for all genes the stop codon was replaced with a valine codon.*




***Supplemental Figure 4.1*** *– Yeast two hybrid interaction matrix on glucose and galactose YPD dropout plates. Full interaction array of B42AD fusion and LexA fusion proteins. Matings were planted on YPD –leu –his –ura –trp with with either glucose or galactose as a carbon source. Glucose does not initiate*

*expression of fusion proteins and represent false positives. Galactose activates expression and growth indicates interaction between proteins of interest.*

# CHAPTER 5 – CONCLUSIONS FUTURE DIRECTIONS

The hermaphroditic flower is purposed to be the ancestral floral form and in this structure we see unparalleled morphologic diversity. The most extreme alteration of floral form is dioecy, the complete segregation of sexual function into separate individuals. The evolution of dioecy from a co-sexual ancestor is thought to be driven by sexual specialization and avoiding inbreeding by favoring outcrossing (Charlesworth & Charlesworth, 1978a; Charlesworth & Charlesworth, 1978b; Lloyd, 1980b). Interestingly, many species that segregate gender function are observed to undergo sex reversals upon hormone application (Korpelainen, 1998). This hormone influenced sexual flexibility is not restricted to dioecious species and different hormones illicit different sexual responses that are species specific. Although monoecious, sex in melons is understood to be influenced by the phytohoromone ethylene and the genes involved and their interactions are well studied. The three genes responsible for sex determination have been elucidated and found to be a WIP-like transcription factor (*CmWIP 1*) and the other two are involved in ethylene biosynthesis (*CmACS-7*, *CmACS-11*) (Boualem *et al.*, 2008; Boualem *et al.*, 2015). A mechanism characterizing their interaction and the resulting sex has been demonstrated (Boualem *et al.*, 2015). However, it remains unclear how these upstream transcription factors and hormone biosynthesis genes interact with or influence the alternative expression of floral organ identity genes to produce unisexual flowers.

Like ethylene in melons, gibberellic acid is important for sex determination in spinach (Chailakhyan, MK & Khryanin, V, 1978a). Unlike melons, the genes responsible for sex determination have not been found but reliable markers have been created (Onodera *et al.*,

2011; Kudoh *et al.*, 2018). Our lab has generated a mechanism linking the GA hormone response pathway and floral organ identity gene expression. We have observed a unique function of spinach B-class genes; in addition to conferring stamen identity, the spinach B class foral identity genes also suppress gynoecium development (Sather *et al.*, 2010). My work has identified a link between the GA signaling pathway and B-class expression (West & Golenberg, 2018). In spinach the expression of a member of the DELLA family of transcription regulators, *SpGAI,* antagonizes B-class expression. When *SpGAI* expression is artificially reduced through either exogenous application of GA or VIGS mediated transient knockdown, B-class expression is elevated, and the individual is masculinized. The inverse situation also holds true, when SpGAI is elevated by exogenous application of a GA-synthesis inhibitor or application of a proteasome inhibitor, B-class genes are suppressed and the individual is feminized. Sampling endogenous *SpGAI* expression from inflorescence tissue we found that females expressed roughly twice as much *SpGAI* when compared to males which agrees with our GA and SpGAI manipulation experiments. This is the first sex determination mechanism directly linking a hormone response gene to the alternative development of floral organs. The alternative expression of SpGAI during flower development seems to act like a switch governing which morphological pathway the individual will traverse. To better understand this process, we investigated gender specific gene expression during floral development and screened potential physical interactions between transcription factors of interest.

To identify the genes required to shape the unique floral morphology of male and female flowers we performed Illumina mediated RNAseq on the inflorescences of each sex. Transcriptome analysis revealed165 contigs that were enriched in male samples and 88

were identified via BLAST, among these were *SpPI* and *SpAP3* whose expression is known to be male specific. Characterization of the male specific transcripts continues along two paths, observation of location, intensity, and time of sex specific gene expression and transient knockdown of sex specific genes. We generated a modified loop-mediated isothermal amplification (LAMP) protocol to apply LAMP detection of specific gene mRNAs to histological sections of male and female inflorescences. This LAMP mediated *in situ* protocol was used to characterize the temporal and tissue specific expression of multiple genes identified in the RNAseq analysis. These data shed light on the expression patterns of gender specific genes that result from sex determination in spinach.

Alternative expression of *SpGAI* results in unisexual flower development. Spinach floral development does not have a transient hermaphroditic phase suggesting *SpGAI* mediated sex determination occurs during the transition to flowering or soon thereafter. LFY is critical for flower development and known to activate the expression of flower organ identity genes (Schultz & Haughn, 1991; Huala & Sussex, 1992; Weigel *et al.*, 1992). Our model suggests that in females the presence of SpGAI specifically inhibits the activation of B-class but not C-class genes. To explore the possibility of interaction between SpGAI and SpLFY we performed yeast two-hybrid and bimolecular fluorescence complementation screens. In both experiments data indicate direct interaction between SpGAI and SpLFY, this represents the first observation of direct interaction between SpLFY and a member of the DELLA family transcription regulators. In *A. thaliana* UFO is known to interact physically with LFY and this interaction is required for proper stamen development (Lee *et al.*, 1997). Both the Y2H and BiFC screens showed evidence of direct interaction between SpLFY and SpUFO. Additionally, the BiFC analysis produced data indicating SpGAI and

SpUFO directly interact. This interaction is particularly interesting as SpUFO is a F-box protein which is a component of the Skp-Cullin-Fbox complex that mediates protein degradation, including DELLA proteins. Taken together and in the context of our mechanism for sex determination a number of possibilities begin to take shape. It is unknown if SpUFO is required for B-class initiation in spinach as it is in *A. thaliana*. If SpUFO truly is required for proper stamen initiation then SpGAI sequestration of this co-factor would allow for specific inhibition of B-class genes without necessarily inhibiting the activation of C-class genes. Although this explains specific B-class inhibition it does not address the interaction observed between SpGAI and SpLFY or the ramifications thereof. The direct interaction between SpGAI and SpLFY would be predicted to sequester SpLFY thus preventing SpLFY from initiating expression of its target genes. Although possible, this direct sequestration motif does not seem plausible as it cannot explain how the SpGAI mediated sequestration of SpLFY prevents the activation of only the B-class genes but not C-class genes, one would expect SpLFY sequestration to prevent activation of both B- and C-class genes. DELLA proteins do not have a canonical DNA binding domain but have been found in ChIP experiments, this is accomplished through intermediaries that bind the target DNA and the DELLA transcription factor (Sun, 2011). In light of this, SpGAI could be localized to the cis-regulatory region of the B-class but not C-class genes through some unknown intermediary. If localized to the B-class regulatory region in this manner SpGAI sequestration of SpLFY would then only affect B-class expression. The interaction between SpGAI and SpUFO could be explained in the context of SpUFOs function in protein degradation. SpGAI, anchored to the cis-regulatory region of B-class genes by an intermediary, binds to and sequesters SpLFY preventing it from activating the B-class

genes, SpUFO could then interact with and target SpGAI for degradation thus releasing SpLFY to initiate B-class expression. However, we observe no allelic difference between SpUFO of male and female spinach and do not have any information on the potential sex specific expression of SpUFO. Alternatively, if SpUFO is truly required for proper activation of spinach B-class genes then SpGAI mediated sequestration independent of SpGAI-SpLFY interaction would provide an additional point of control for SpGAI in the activation of B-class genes. The physical interactions observed do not force any major alterations of our purposed mechanism for sex determination in spinach but provide opportunity to further refine and clarify this regulatory architecture.

To improve upon this body of work several experiments should be considered. Further refinement of our model of sex determination requires *in vitro* confirmation of SpGAI, SpFLY, and SpUFO interactions as well as *in planta* observations of all combinations performed in the yeast two-hybrid screen. Additionally, the *in vitro* analysis can also incorporate cis-regulatory regions of both B-class genes to assess the requirement of native DNA for any interactions. Pull down and identification of any proteins that associate with the cis-regulatory region of B-class genes may help clarify how *SpGAI* specifically prevents activation of these genes but not C-class targets. Further assessment of the unique gynoecium suppression function previously observed in spinach B-class genes would help develop our model. Utilizing CRISPER-Cas9 technology and *A. thaliana* transgenic protocols it is possible to replace *AtAP3* and *AtPI* with spinach varieties and observe spinach B-class influence of *A. thaliana* gynoecium development. Given the depth of research regarding *A. thaliana* flower development any perturbation of gynoecium development may have been observed before and could provide crucial insight to the gene

targets of SpAP3 and SpPI involved in carpel suppression. Additionally, the transcriptomes of the *SpAP3* and *SpPI* transgenics can be compared to published *A. thaliana* transcriptomes to identify any enriched or depleted genes. The reciprocal experiment, transgenic spinach harboring *A. thaliana* B-class genes would certainly benefit our understanding of alternative sex development. However, this would likely be much too time consuming to achieve as spinach transgenics are notoriously difficult and unlike *A. thaliana* there are no *SpAP3* or *SpPI* mutant lines available thus they would have to be created, not to mention proper CRISPER-Cas9 mediated insertion of *AtAP3* and *AtPI*.

To advance our understanding of unisexual gene expression patterns in spinach a functional analysis must be undertaken. Our current transcriptome is biased to identify overrepresented transcripts from male but not female samples. Our model suggests sex determination functions in a switch-like manner, in which C-class gene expression initiates gynoecium development genes in females while in males, B- and C-class organ identity gene expression is initiated however, androecium genes then suppress gynoecium development. Given this theory, we do not expect female specific genes with male suppressing function to be required for unisexual development, although our theory does not preclude this possibility. However, our theory does require that B-class genes themselves, or some downstream gene that requires B-class activity have some gynoecium suppressing function and therefore could be found within our admittedly biased sample.

The spinach transcriptome we produced found 165 transcripts overexpressed in male tissues and 88 were identified via BLAST search. To advance this gynoecium suppressor hunt we should attempt to identify the remainder of the overrepresented transcripts as

well as begin a mass cloning strategy of all 165 genes into our VIGS vector pWSRi. Currently, 16 of the 165 genes have primers designed for pWSRi insertion and a fraction of the 16 have already been cloned into pWSRi as well as into pGEM T-ez. Designing primers for the remaining genes and cloning into at least pWSRi would be an achievable, although time consuming goal. It might be more time efficient to modify our pWSRi vector with BP/LR recognition domains thus allowing the use of the highly efficient gateway cloning method or perhaps more accurately, avoiding the hassles of restriction-ligation based cloning. Once accomplished transient knockdown of all male-overrepresented genes becomes a possibility, any knockdown that fails to suppress the gynoecium would therefore be implicated in the process of carpel inhibition. Additionally, reproducing male and female transcriptomes might be beneficial. The initial results were male-biased and the tissues used to produce mRNA were harvested from flowers at different stages of development. If RNAseq were to be repeated the developmental stage of the flower should be considered and young flowers prioritized however, this could be problematic as sequencing methods require samples have some minimum concentration. Although RNAseq technology has improved significantly and the minimum concentrations required have been reduced, methods to maximize RNA yield as well as amplify mRNA *in vitro* should be considered.

Why study the evolution and development of dioecy? Compared to mammals, dioecy evolved quite recently in plants and evolved multiple times independently which allows a unique opportunity to explore multiple pathways that resulted in the evolution of unisexuality. The ability to study the requirements and ramifications of unisexuality in the evolution of plant sex chromosomes could shed light on the evolution of sex chromosomes

in mammals and other unisexual organisms that we cannot test directly. Identifying and analyzing the chromosomal regions responsible for sex determination and elucidating the genes that reside within these regions are critical for understanding the evolutionary history of sexual segregation. In angiosperms sex determination via differential regulation appears to be common amongst monoecious and many dioecious species. However, there is no complete mechanism linking confirmed sex determination genes to the differential expression of floral organ identity genes or genes responsible for sex organ suppression. Understanding the multiple ways this process can be achieved is an important aspect of our knowledge of plant development. The mechanism for sex determination in spinach hypothesized here provides a regulatory framework that can explain previous observations and suggests a direct link between the GA hormone response pathway and floral development pathway. However, we do not inform nor speculate about the identity of the sex determining gene(s). Once identified it will be of great interest to challenge our mechanism for unisexual development in the context of the gene(s) responsible for sex determination.

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**ABSTRACT**

**A MECHANISM FOR SEX DETERMINATION IN DIOECIOUS CULTIVATED SPINACH**

by

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While unisexual flowers have evolved repeatedly throughout angiosperm families, the actual identity of sex determining genes has been elusive, and their regulation within populations remains largely undefined. Additionally, sex liability is often observed in unisexual plants and has been correlated to external and internal cues, suggesting that the genes responsible for unisexual morphology are not necessarily segregating but rather differentially regulated. Understanding these processes will be of significant theoretical and agronomical importance.

Cultivated spinach is a dioecious species in which an individual will bear alternative sexual organs. Previous work has identified spinach B class floral organ identity genes, *SpAP3* and *SpPI,* to have a novel function resulting in the suppression of gynoecium development. We begin by testing the mechanism of the feminization pathway and its relationship to masculinization. Our results confirm earlier observations that exogenous applications of the plant hormone GA masculinize female flowers. Furthermore, inhibition

of GA production and of proteasome activity feminizes male flowers. These observations are consistent with the role of the GA in spinach sexual development however, when assessing the GA content of male and female inflorescences and flowers we observe no significant difference between the sexes. We isolate and describe a single DELLA gene (*SpGAI*) in spinach. DELLA proteins are repressive transcription factors responsive to GA. Gene silencing of *SpGAI* in females allows activation of B class floral identity genes, and hence masculinization of female flowers. Additionally, *SpGAI* is differentially expressed in female versus male flowers. These results strongly implicate the role of SpGAI as a feminizing factor in spinach and suggest that the feminizing pathway is epistatic to the masculinizing pathway. We present a unified model for alternative sexual development in spinach and discuss the implications of such a model to established theory.

Our model predicts an interaction between *SpGAI* and *SpLFY,* a key transcription regulator involved in the transition from vegetative to floral growth. To explore this potential interaction, we used Yeast 2 Hybrid *in vivo* and Bimolecular Complementation *in planta* to screen for physical interaction. Preliminary results indicate a physical interaction occurs between SpGAI and SpLFY. The aforementioned genes and processes address the initial steps of sex determination in spinach. To begin characterization of genes that are important for morphogenesis of unisexual flowers, we generate a transcriptome from male and female inflorescences. Analysis revealed 165 differentially expressed transcripts, of which 88 could be identified by BLAST. Candidate genes were chosen, and differential expression was confirmed with qRT-PCR analysis. Loop-mediated isothermal amplification (LAMP) based *in situ* observation of genes identified as male specific as well as female specific were performed on spinach inflorescence sections. The sex specific expression detected with *in silico* analysis

was confirmed *in vitro* with qRT-PCR and *in planta* with LAMP mediated gene expression observation.

These studies represent important contributions to our understanding of sexual development in unisexual angiosperms. There has been much difficulty identifying sex determining genes and despite great effort no such genes have been described in spinach. Our work identified differential SpGAI expression as critical for unisexual development and altering that expression through various methods has predictable results. Combined with data previously generated in our lab we present a mechanism linking SpGAI expression and flower organ identity gene expression resulting in unisexual flowers. The transcriptome and list of differentially expressed genes will be a useful resource to identify the genes responsible for the morphological differentiation between the sexes. In the future candidate genes will be selected for functional testing using the VIGS based approach developed previously in our lab.

# AUTOBIOGRAPHICAL STATEMENT

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### Education

Schoolcraft Community College, Livonia MI 48128

Associates of Science in Biological Sciences

Wayne State University, Detroit MI 48202

Bachelor of Science in Biological Sciences

### Publications

Podushkina, D., West, N.W. and Golenberg, E.M., 2019. Utilizing multiplex fluor LAMPs to illuminate multiple gene expressions in situ. *PloS one*, *14*(10).

West, N.W. and Golenberg, E.M., 2018. Gender-specific expression of GIBBERELLIC ACID INSENSITIVE is critical for unisexual organ initiation in dioecious Spinacia oleracea. *new phytologist*, *217*(3), pp.1322-1334.

Golenberg, E.M. and West, N.W., 2013. Hormonal interactions and gene regulation can link monoecy and environmental plasticity to the evolution of dioecy in plants. *American Journal of Botany*, *100*(6), pp.1022-1037.

### Select Oral Presentations

Feminization Pathway Mediated by GAI Repressor Protein in *Spinacia oleracea* L.

Botany 2015 Conference Edmonton, Canada

### Select Poster Presentation

Characterization of male specific gene function in unisexual crop *Spinacia oleracea* L.

Wayne State University Biological Sciences Departmental Retreat 2017

### Select Awards and Honors

Dennis P. Smith Award – Excellence in Genetics Research

Graduate Research Award

Graduate Teaching Award – Genetics