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EVOLUTION OF DUPLICATED *HAN*-LIKE GENES IN *PETUNIA x HYBRIDA*.

A Thesis Presented

by

Beck Powers

to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements
for the Degree of Master of Science
Specializing in Plant Biology

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ABSTRACT

Gene duplications generate critical components of genetic variation that can be selected upon to affect phenotypic evolution. The angiosperm GATA transcription factor family has undergone both ancient and recent gene duplications, with the *HAN*-like clade displaying divergent functions in organ boundary establishment and lateral organ growth. To better determine the ancestral function within core eudicots, and to investigate their potential role in floral diversification, I conducted *HAN*-like gene expression and partial silencing analyses in the asterid species petunia (*Petunia* x *hybrida*). My results indicate duplication of *HAN*-like genes at the base of Solanaceae followed by expression diversification within the flower. Although no aberrant phenotypes were apparent following single gene knockdowns, silencing of both paralogs lead to leaf senescence. Together with other functional studies, these data suggest a possible ancestral role for *HAN*-like genes in core eudicot shoot apical meristem development, followed by functional diversification following both speciation and duplication.

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CHAPTER 1: COMPREHENSIVE LITERATURE REVIEW

1. Introduction

Organisms display incredible variation in structure and function, and these elements of phenotype are critical to the processes of diversification and speciation. The field of evolutionary developmental biology, or "evo-devo," seeks to understand the types of genetic and developmental changes that affect phenotype diversity. Work in both animal and plant systems has shown that, despite immense diversity in organismal form, many genes are conserved amongst species. By studying the evolutionary history of genetic pathways across various organisms in the phylogeny of life, we can begin to understand how the great majority of morphological and functional diversity, in the words of Charles Darwin, "have been, and are being, evolved". This process usually starts off with a detailed investigation of development in a few model species, followed by comparative analyses in phenotypically divergent taxa. This review examines the genetic underpinnings of angiosperm vegetative and floral morphogenesis in an evolutionary and developmental context.

2. Angiosperm Postembryonic Morphogenesis

In "higher" plants, post-embryonic growth of the aerial plant body is carried out by the continuous activity of a group of undifferentiated mitotic cells, collectively known as the shoot apical meristem (SAM). The angiosperm SAM is typically composed of three functionally distinct zones: a central zone of gradually dividing stem cells, a peripheral zone of rapidly dividing central zone daughter cells, and a rib zone from which internal

stem tissues are derived (Steeves and Sussex, 1989; Bowman and Eshed, 2000). Throughout development, the pluripotent cells of the SAM give rise to differentiated lateral organs, including axillary meristems (that give rise to branches) and subtending leaves (Steeves and Sussex, 1989; Meyerowitz, 1997; Barton, 1998; Bowman and Eshed, 2000).

Several genetic components required for both the establishment and maintenance of aerial meristem identity have been identified, most notably the homeodomain transcription factor *WUSCHEL* (*WUS*), which is expressed in the subapical central zone of the *Arabidopsis thaliana* SAM (Laux et al., 1996; Mayer et al., 1998; Lenhard and Laux, 1999). In plants with mutations in *WUS*, the SAM is initially formed but soon arrests after production of only a few lateral organs (Laux et al., 1996). The undifferentiated state of cells in the central zone of the *A. thaliana* SAM is controlled by the combinatorial action of the *KNOX* gene, *SHOOT MERISTEMLESS* (*STM*), and NAC transcription factor paralogs *CUP-SHAPED COTYLEDON1* (*CUC1*), *CUC2*, and *CUC3* (Barton and Poethig, 1993; Endrizzi et al., 1996; Long et al., 1996; Aida et al., 1999; Vroemen et al., 2003). Specifically, *CUC1* and *CUC2* function redundantly in the upregulation of *STM* expression in the SAM, but not developing organ primordia, thereby defining SAM identity (Long et al., 1996; Aida et al., 1999). In turn, *STM* directly upregulates *CUC1* and indirectly activates *CUC2* and *CUC3* to promote meristem-organ boundary establishment (Spinelli et al., 2011).

3. Lateral Organ Boundary Establishment

An underlying mechanism in the formation of new lateral organs from a meristem is the establishment of a specialized boundary that divides newly initiated organ primordia from meristematic activity (Aida et al., 1997; Aida and Tasaka, 2006). Boundary formation between meristem and organ primordia requires a reduction in cell division, which has been observed both morphologically (Hussey, 1971) and at the cellular level (Breuil-Broyer et al., 2004). While cells of the meristem and organ primordia are round and either flat or convex, boundary cells are concave in shape due to the mechanistic forces of boundary cell elongation and contraction (Hussey, 1971; Kwiatkowska and Dumais, 2003; Kwiatkowska, 2004; Reddy et al., 2004). Cells at the boundary of *A. thaliana* inflorescence and floral meristems show a lack of DNA synthesis and reduced expression of cell-cycle genes (Breuil-Broyer et al., 2004).

Several transcriptional regulators of boundary formation have been identified, most notably the aforementioned NAC transcription factors *CUC1/2/3*. Orthologs of *CUC* genes in other plants include the *NO APICAL MERISTEM* (*NAM*) gene in *Petunia hybrida* (petunia) and *CUPULIFORMIS* (*CUP*) in *Antirrhinum majus* (snapdragon). The *A. thaliana* double *cuc1*:*cuc2* mutant and petunia *nam* mutants display fusion of embryonic leaves and of some floral organs, whereas *cup* mutants show fusion in nearly all above-ground lateral organs (Souer et al., 1996; Aida et al., 1997; Takada et al., 2001; Vroemen et al., 2003; Weir et al., 2004). Interestingly, NAC genes are expressed at the boundary between the meristem and lateral organs (M-O boundary) and between adjacent lateral organs (O-O boundary). The improper separation of organs in NAC mutants and expression of NAC genes at the M-O and O-O boundaries collectively suggests a role in

maintenance of boundaries between meristem and lateral organs, and amongst lateral organs themselves (Aida and Tasaka, 2006).

4. Origin Of Flowers And Diversification Of The Floral Ground Plan

With over 350,000 documented species, the flowering plants (angiosperms) represent the most diverse lineage of vascular land plants on earth (Paton et al., 2008). The reproductive body of angiosperms, the flower, is arguably one of the most important morphological innovations to arise in the plant kingdom (Willis and McElwain, 2013), yet the selective pressures that drove its evolution are largely unknown. One hypothesis suggests that the flower evolved to attract insect pollinators, thus advancing the exchange of genetic material among widely dispersed early angiosperm individuals and therefore increasing overall plant fitness (Willis and McElwain, 2013). Subsequent diversification of the major clades of angiosperms occurred quite rapidly during the mid-Cretaceous (also known as Darwin's "abominable mystery"), resulting in immense variation in morphology, particularly in the flower (Moore et al., 2007).

In their vastly different forms, flowers share a common ground plan composed of both fertile (androecium and gynoecium) and sterile (perianth) organs that originate as lateral organ primordia from the floral meristem in a mostly centripetal fashion (Endress, 2006). A major trend throughout angiosperm evolution is the elaboration of the floral ground plan through the process of synorganization, or the intimate integration (e.g. fusion) of floral elements into functional modules (Endress, 2006, 2015; Armbruster et al., 2009; El Ottra et al., 2013). The whorled floral phyllotaxis of most angiosperms is a

prerequisite for synorganization of floral elements by fusion, such that adjacent organs within a whorl are circumferentially tangential and therefore more likely to unite (Endress, 2015). Examples of this kind of synorganization can be found in all four floral whorls: synsepaly, sympetaly, synstemony, and syncarpy (Endress, 2015).

Sympetaly, the fusion of petals into a corolla tube, generates tremendous potential for diversification in floral form (Endress, 2011) and restricts access to pollinator rewards that can result in canalized and efficient pollination, thereby increasing plant fitness. Such a petal configuration also protects inner male and female organs during floral development and provides a structurally stable and attractive guide for pollinators (Endress, 2001). Development of petal fusion requires unison of primordial petal meristems (areas of active cell division) at the tip of and between growing petals (Specht and Howarth, 2015). Historically, sympetaly has evolved independently in multiple angiosperm lineages (e.g. Dioscoreales, Ranunculales, Saxifragales, and Orchidaceae) but the most impressive lineage with ancestrally fused petals is the diverse Asteridae lineage of core eudicots (Endress, 2011). The asterids are an immensely diverse group of plants, comprising over 25% of all flowering plant diversity (*sensu* APG III, 2009). Evolution of sympetaly is thus hypothesized to serve as a major driving force in the rapid diversification of this successful flowering plant lineage.

5. Role Of Boundaries In Floral Development

During the reproductive phase of the plant life cycle, the SAM transitions into an inflorescence meristem, which in turn, gives rise to individual determinate floral meristems (Steeves and Sussex, 1989). Similar to the SAM and inflorescence meristem,

the floral meristem maintains a central population of pluripotent and peripheral cells that differentiate into the various lateral organs of the flower (sepals, petals, and stamens). However, unlike other aerial meristems, the floral meristem itself eventually differentiates and terminates in the female reproductive organs (gynoecium).

Despite immense morphological variation, the relative position of floral organs is largely conserved across the angiosperms. During a flowering plant's reproductive phase, a floral meristem typically gives rise to four concentric whorls of dome-shaped organ primordia that will acquire a specific identity: sepals (whorl 1), petals (whorl 2), androecium (whorl 3), and gynoecium (whorl 4). Floral organ identity within each individual whorl is dictated by the position-specific and combinatorial expression of MADS-box transcription factors, as described by the ABC model of floral development (Bowman et al., 1989; Coen and Meyerowitz, 1991). The basic principle of this model, based on phenotypes of homeotic mutants, proposes that the expression of each gene in a given whorl specifies the organ identity of that whorl: the first whorl by A class genes alone, specifying sepal identity, the second whorl by A and B class genes together, specifying petal identity, the third whorl by B and C class genes together, specifying stamen identity, and the fourth whorl by C class genes alone, specifying gynoecium identity (Coen and Meyerowitz, 1991).

While the ABC model explains how floral organ identity is established, it cannot fully explain how boundaries are formed between and within floral whorls. A plethora of other transcription factors have been identified as regulators of boundary formation between and within floral whorls. In the *A. thaliana* flower, boundary formation is fulfilled in part by *CUC* genes encoding NAC transcription factors. In the developing

flower, *CUC* genes are expressed between the bases of floral organ primordia where cell proliferation is reduced (Breuil-Broyer et al., 2004). The loss of this expression in the double *cuc1:cuc2* mutant results in fusion within the sepal and stamen whorls, suggesting that *CUC1* and *CUC2* are redundantly required for proper delimitation of organs in these floral whorls (Ishida et al., 2000; Takada et al., 2001).

6. Candidate Genes In The Evolution Of Sympetaly

Several gene families are proposed candidates in the evolution of sympetaly, based on our understanding of the genetic control of organ boundary establishment and lateral organ expansion in free-petaled (choripetalous) *A. thaliana*. A potential genetic component in the evolution of asterid sympetaly is the WUSCHEL-like homeobOX (WOX) family of plant-specific transcription factors. WOX genes play essential roles in key processes of plant development across angiosperms, including maintenance of shoot and root apical meristems (Sarkar et al., 2007; Nardmann and Werr, 2009), embryonic polarity patterning (Haecker et al., 2004), and lateral organ development (Matsumoto and Okada, 2001; Vandenbussche et al., 2009; Tadege et al., 2011; Niu et al., 2015). In monocots such as rice (*Oryza sativa*) and maize (*Zea mays*), *WOX3* promotes lateral outgrowth of leaves while in the eudicot *A. thaliana*, *WOX3* promotes lateral expansion of floral organs, including petals (Matsumoto and Okada, 2001). Of particular interest is the *Medicago truncatula* (barrel medic) *WOX3* ortholog, which promotes proper fusion of lateral and ventral organs of the petal whorl (Niu et al., 2015). In the sympetalous asterid species petunia, the WOX1 clade gene *MAEWEST* (*MAW*) acts as a promoter of lateral outgrowth in lateral organs. Mutations in this gene result in reduced leaf size and largely

unfused petals (Vandenbussche et al., 2009), suggesting that it may be a key component of the petal fusion pathway. At least three other WOX genes have been found in the petunia genome (Costanzo et al., 2014) but it is unknown whether these orthologs function similarly to *MAW* in the control of sympetaly.

Another group of transcriptional regulators potentially involved in the transition from free to fused petals are GATA zinc finger transcription factors. GATA factors comprise an evolutionarily conserved eukaryotic protein family of developmental regulators possessing a class IV zinc finger ($CX_2CX_{17-20}CX_2C$) that preferentially binds to the DNA consensus sequence (A/T)GATA(A/G) (Lowry and Atchley, 2000). In plants, approximately 30 genes code for GATA transcription factors which can be subdivided into four subfamilies based on exon-intron structure and sequence similarity of the conserved DNA-binding domain (Reyes et al., 2004). Plant GATA factors play diverse roles in both development and physiology, including SAM organization and proliferation (Zhao et al., 2004), seed germination (Liu et al., 2005), and chloroplast biogenesis (Chiang et al., 2012). The *A. thaliana* subfamily-II GATA factor *HANABA TARANU* (*HAN*) is required for positioning of the proembryo boundary during embryogenesis (Nawy et al., 2010) and is later required for the establishment of boundaries between the SAM and lateral organ primordia (Zhao et al., 2004).

During the *A. thaliana* reproductive phase, *HAN* functions in the formation of the boundary between the inflorescence and floral meristems and further functions in boundary formation between floral whorls (Zhao et al., 2004). Recent work suggests that *HAN* fulfills its role as a transcriptional regulator of the floral meristem-organ (M-O) boundary by communicating both with meristem-specific genes *ARGONAUTE*

10/*PINHEAD* (*PNH*) and *KNAT1*/*BREVIPEDICELLUS* (*BP*), and organ primordia-promoting genes *JAGGED* (*JAG*) and *BLADE-ON-PETIOLE 2* (*BOP2*) (Ding et al., 2015).

In monocots, *HAN* orthologs *TASSEL SHEATH 1* (*TSH1*) in maize, *NECK LEAF 1* (*NL1*) in rice, and *THIRD OUTER GLUME* (*TRD*) in barley all play a conserved function in the suppression of bract outgrowth (Wang et al., 2009; Whipple et al., 2010). A *HAN*-like gene recently characterized in the rosid eudicot cucumber (*Cucumis sativus*) regulates SAM development by potentially mediating the expression of genes involved in meristem establishment and maintenance, including *WUS*, *STM* and *BP* (Ding et al., 2015). However, misexpression of *CsHAN* causes stunted seedling growth, fewer flowers, and lobed leaves (Ding et al., 2015), suggesting a divergent role compared to *A. thaliana HAN* in determining lateral organ number and shape.

7. Gene Duplication And Diversification In Plants

In plants, single gene duplication as well as duplication of the entire genome is widespread and occurs fairly frequently, creating large families of paralogous genes (Panchy et al., 2016). In flowering plants alone, whole genome duplication (WGD) has occurred multiple times over the course of their 200 million years of evolution (Simillion et al., 2002; Shiu et al., 2005; Cui et al., 2006; Soltis et al., 2007; Lyons et al., 2008; Van de Peer et al., 2009). Interestingly, transcription factor families have the highest expansion rate compared to paralogous families in other eukaryotes, likely due to higher rates of genome duplication in plants (Shiu et al., 2005). Furthermore, it has also been

shown that transcription factor families have expanded in parallel in phylogenetically distant plant species (Shiu et al., 2005; Panchy et al., 2016).

Retention in the genome without change in function is a common fate of duplicate genes, either because there is selection for functional redundancy (Zhang, 2012) or simply because not enough time has passed to allow for the accumulation of deleterious mutations and subsequent loss of ancestral function in one paralog (genetic drift) (Panchy et al., 2016). Alternatively, fixation of duplicate genes can occur when maintaining both genes in the genome confers fitness advantages such as increased gene product (Ohno, 1970) or partitioning of the ancestral function between paralogs (subfunctionalization) (Force et al., 1999). Furthermore, duplicate genes may acquire a new molecular function (neofunctionalization), such as change in expression patterns that may lead to novel phenotypes that may or may not end up being adaptive.

8. Duplication And Diversification Of Eudicot *HAN*-Like Genes

The aforementioned plant GATA-3 transcription factors have undergone both ancient and more recent gene duplications in plants, and are implicated in lateral organ growth and boundary establishment (Reyes et al., 2004). In GATA-3 genes containing the HAN motif (henceforth, *HAN*-like genes), both conservation and diversification of ortholog function across angiosperms has been observed. The focus of my thesis is to investigate whether diversification in *HAN*-like genes was essential for shifts in floral morphology in the asterid *Petunia x hybrida*.

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CHAPTER 2: DUPLICATION AND DIVERSIFICATION OF *HAN*-LIKE GENES IN *PETUNIA x HYBRIDA*

INTRODUCTION

Gene duplication is common in plants and, like other mutational events, contributes to standing variation that is party to different types of selection (Blanc and Wolfe, 2004; Ambrosino et al., 2016). The most common long-term fate of a paralog is loss through the accumulation of deleterious mutations (pseudogenization) (Ohno, 2013). This can either occur through neutral processes related to functional redundancy, or selection against gene dosage effects (Conant et al., 2014). Less often, duplicate genes are retained in the genome, mostly through partitioning of ancestral gene function between the two descendants (subfunctionalization) (Force et al., 1999; Freeling et al., 2015), or the acquisition of a new function by one paralog (neofunctionalization) (Hughes, 1994; Van de Peer et al., 2009). Subfunctionalization has been implicated in the loss of pleiotropic constraints (Guillaume and Otto, 2012), allowing correlated traits to evolve independently, whereas neofunctionalization can directly generate phenotypic novelties (Zhang, 2006). Understanding the impacts of duplication and retention events is important to defining the genetic basis of trait evolution, and the extent to which plant gene networks are conserved (Panchy et al., 2016).

GATA3-type transcription factors comprise a large gene family that has expanded through both ancient and recent gene duplications, with 29 and 30 members in the genomes of the monocot *Oryza sativa* (rice) and eudicot *Arabidopsis thaliana*, respectively (Reyes et al., 2004). GATA3 genes have previously been subdivided into four classes (A through D) based on sequence similarity of the DNA-binding domain,

exon-intron structure, and the presence of additional protein domains (Reyes et al., 2004). Within the B-class GATA3 clade, two subclasses of genes have been categorized based on the presence of either a HAN- (Reyes et al., 2004) or LLM- (leucine-leucine-methionine) domain (Behringer et al., 2014). The HAN domain, which has yet unknown function, was first described in the *A. thaliana* B-class GATA gene *HANABA TARANU* (*HAN*), and is also present in two closely related paralogs *HAN*-like 1 (*HANL1*) and *HAN*-like 2 (*HANL2*) (Zhao et al., 2004).

A. thaliana HAN is expressed broadly in vegetative and reproductive tissues, and more specifically in boundary regions between meristems and nascent lateral organs, such as cotyledons, leaves, and floral organs (Zhao et al., 2004). Consistent with patterns of expression, *han* mutants have flattened meristems that are reduced in size, with meristem perturbation becoming progressively worse from vegetative to reproductive development. In early development, *han* mutants have slightly misshapen embryos and partially fused cotyledons, whereas during reproductive development the four floral whorls are strongly affected in terms of their arrangement (phyllotaxy), decreased organ number, and concomitant within-whorl organ fusion (Zhang et al., 2013). It was recently demonstrated that HAN is a negative regulator of many genes involved in cell division and expansion, including itself and *HANL2*, and affects the expression of several genes involved in floral organ development and hormone action (Zhang et al., 2013). Despite *hanl2* single mutants having no obvious phenotype, double *han:hanl2* mutants show phenotypes more extreme than *han* plants. These data suggest at least partial redundancy for *han* and *hanl2* in organ boundary establishment and as negative regulators of growth; little is currently known about the function of the other close paralog *hanl1*.

In addition to altered functionalization following gene duplication, functional evolution can occur between orthologs following speciation. In the case of B-class GATA genes containing the HAN motif (hereafter *HAN*-like genes), evidence across angiosperm species suggests a combination of functional conservation and diversification of orthologs. In monocots such as maize (*Zea mays*), rice (*Oryza sativa*), and barley (*Hordeum vulgare*), *HAN*-like genes *TASSEL SHEATH 1* (*TSH1*), *NECK LEAF 1* (*NL1*), and *THIRD OUTER GLUME* (*TRD*), respectively, function to repress outgrowth of subtending leaves (bracts) of the floral inflorescence (Wang et al., 2009; Whipple et al., 2010). Similar to *HAN*, a *HAN*-like gene recently characterized in cucumber (*Cucumis sativus*, Cucurbitaceae), a rosid eudicot like *A. thaliana*, regulates shoot apical meristem (SAM) development by mediating the expression of genes involved in meristem establishment (Ding et al., 2015). However, both upregulation and downregulation of *CsHAN* expression results in retarded seedling growth, fewer flower buds, and lobed leaves (Ding et al., 2015), suggesting a different role for this gene compared to *A. thaliana HAN* in determining lateral organ number and shape. The scarcity of functional data for *HAN*-like genes in core eudicots outside rosids, combined with the lack of SAM and floral phenotypes in monocot *HAN*-like mutants, makes it difficult to infer the extent and direction of evolutionary change in this gene lineage across angiosperms. Moreover, it is unclear whether diversification in the *HAN*-like gene lineage has been important for shifts in both leaf and flower morphology.

To address these questions, we conducted functional analysis on *HAN*-like transcription factors in the horticulturally important asterid core eudicot species petunia (*Petunia x hybrida*). Asterids are sister to rosids and comprise ~25% of angiosperm

diversity (Bremer et al., 2009). Unlike *A. thaliana* and cucumber, petunia petals are congenitally fused into a corolla tube, which has been implicated in pollinator specialization (Stuurman et al., 2004; Knapp, 2010; Endress, 2011). It is hypothesized that the fusion of petals into a tube-like structure is due to either the suppression or loss of organ boundary genes at the margin of petal primordia (Zhong and Preston, 2015; Zhong et al., 2016). To determine the role of petunia *HAN*-like genes in SAM, leaf, and flower development, we used a combination of gene expression and partial gene-silencing. Our data suggest that a duplication of *HAN*-like genes occurred at the base of the Solanaceae and that the resultant paralogs function redundantly in promoting overall plant growth, possibly through conserved regulation of the SAM, but are either not involved or are redundant with other factors in affecting leaf and flower organogenesis.

MATERIALS AND METHODS

Gene isolation

Genes used in this study were amplified from either genomic DNA (*PhGATA19*) or cDNA (*PhGATA18* and *DcGATA18*). Genomic DNA was extracted from petunia leaves using the Edwards method as previously described (Edwards et al., 1991; Tamari et al., 2013). Total RNA was extracted from carnation (*Dianthus caryophyllus*) and petunia vegetative tissues, inflorescences, and dissected flowers using the TriReagent (Life Technologies) method, followed by treatment with TURBO DNase (Life Technologies) to remove contamination with genomic DNA according to the manufacturer's instructions. Synthesis of cDNA from 1μg of RNA template was performed using SuperScript reverse transcriptase (BioRad) followed by dilution to the standard cDNA

concentration of 1:10. Degenerate forward primers based on Solanaceae *GATA18*-like and *GATA19*-like HAN motif genes were used in combination with the universal poly(T) adaptor reverse primer (cDNA) and/or a degenerate reverse primer (gDNA) to amplify petunia *HAN*-like genes (Appendix 1). Purified PCR products were ligated to pGEM-T vector (Promega) and transformed into *E. coli* DH5-α competent cells. Eight colonies per amplicon were used for PCR amplification with universal M13 primers, and sequenced with universal T7 and SP6 primers at Beckman Coulter Genomics (Danvers, MA) to verify identity.

Identifying *HAN*-like orthologs using phylogenetic analyses

To infer the evolutionary relationships of angiosperm *HAN*-like genes, a phylogenetic tree was created. *HAN*-like genes from representative angiosperms were downloaded from NCBI (https://www.ncbi.nlm.nih.gov/), Carnation DB (http://carnation.kazusa.or.jp/ (Yagi et al., 2014)), and the SolGenomics Network (https://solgenomics.net/; (Fernandez-Pozo et al., 2015) following a BLAST search using *A. thaliana HAN* (At3g50870) as the query. Amino acid sequences of the highly conserved GATA domain and HAN motif (Behringer and Schwechheimer, 2015) were manually aligned using Mesquite (ver 3.04) (Maddison and Maddison, 2015), and Bayesian inference performed on nucleotide sequences using MrBayes 3.2.6 (Ronquist et al., 2012) on XSEDE, applying the JModetest2-recommended GTR+G substitution model (Darriba et al., 2012). The Markov chain Monte Carlo permutation of tree parameters was executed for two runs of 10,000,000 generations each with trees sampled every 1000 generations. Stationarity was determined for each run by plotting each run's log-likelihood scores against generations

using Tracer v1.6.0 (Rambaut et al., 2013). The first 1,000,000 generations were discarded as the burn-in phase. The 50% majority rule consensus tree was calculated from the remaining sampled trees and viewed in FigTree v1.4.2 (Rambaut and Drummond, 2014). Posterior probabilities were obtained using MrBayes v3.2.6 (Ronquist et al., 2012). Maximum likelihood analysis was also performed using RAxML 8.2.4 (Stamatakis, 2014) on XSEDE with 1000 bootstrap replicates. Tree search analyses were performed on the aligned matrix using GTR+G nucleotide substitution model as recommended by jModelTest2 using Akaike Information Criteria (AIC) (Darriba et al., 2012).

Gene expression analyses

To determine if *A. thaliana HAN* orthologs are similarly expressed in carnation (a basal asterid with unfused petals) and petunia (with derived fused petals), samples for gene expression analysis were collected from roots, stems, leaves, 10 mm floral buds, and dissected floral organs from 10 mm flower buds. For petunia, additional samples were obtained from the petal lobes, transition zone, upper corolla tube, and lower corolla tube of 10 mm flowers to determine if gene expression varied across the fused and unfused portions of the corolla (Figure 2E). Primer pairs for quantitative reverse transcriptase (qRT)-PCR were designed using Primer3 (Rozen and Skaletsky, 2000) for focal carnation and petunia genes *DcGATA18*, *PhGATA18*, and *PhGATA19* (Appendix 1), and tested for efficiency using Fast SYBR® Green Master Mix (Life Technologies) as previously described (Preston and Hileman, 2010). Using efficiency-corrected calculations, triplicate *DcGATA18*, *PhGATA18*, and *PhGATA19* transcript levels were normalized to the

geomean of *EF1alpha* and *UBQ5* housekeeping gene expression (Mallona et al., 2010), and analyzed using the delta delta C_T method (Pfaffl, 2001). Analyses were conducted on different vegetative and reproductive cDNAs, each with three technical and at least three biological replicates.

Vector construction and plant transformation

To functionally characterize petunia *GATA18* and *GATA19*, two virus-induced gene silencing (VIGS) constructs were created based on different regions of the gene that were cloned into an empty tobacco rattle virus 2 (TRV2) vector. The first construct contained a 108 bp region of the *PhGATA18* 3'-UTR region (henceforth TRV2:*PhGATA18utr*), whereas the second construct contained a 243 bp region of *PhGATA18* spanning 34% of the conserved GATA domain (henceforth TRV2:*PhGATA18code*). Gene fragments were amplified from petunia floral bud cDNA using gene-specific primers modified to add restriction fragment (*Bam*HI and *Xho*I) sequences to either end (Appendix 1 and 2), sequence-verified, cloned into TRV2, and transformed into electrocompetent *Agrobacterium tumefaciens* strain EHA105. As an experimental control, we used a 194 bp fragment of the petal pigment gene *CHALCONE SYNTHASE* (*CHS*) cloned into TRV2 as previously described (Chen et al., 2004; Preston et al., 2014).

For plant transformation, single colonies of each TRV2 vector and the TRV1 replication vector were grown overnight on LB plus antibiotic agar plates at 28°C, and TRV2 constructs were PCR screened for presence of each construct using previously described primers pYL156F and pYL156R (Hileman et al., 2005). Positive cultures were used to inoculate 350 mL LB and grown overnight, pelleted, and resuspended in

infiltration medium to an OD_{600} of 0.8-1.0. Each construct was incubated at room temperature for at least four hours before infiltration. Each TRV2 construct was mixed in equal proportions with TRV1 and infiltrated into half the leaves of 18-27 petunia plants at the four to six leaf, pre-anthesis, and post-anthesis stages. Approximately three to four weeks post-infiltration, plants were PCR screened for presence of their corresponding TRV2 vector and monitored for overall growth, leaf fusion, floral organ fusion, and floral organ number.

Verification of construct specificity

To test for the possibility of non-specific silencing by the *GATA18* gene constructs, we used a script that mapped all possible contiguous 21-bp fragments of the target gene to petunia leaf, floral, and inflorescence transcriptomes (Villarino et al., 2014) using Bowtie 1.01 (Langmead et al., 2009). We followed up this analytical approach by comparing expression levels of *PhGATA18* and *PhGATA19* between TRV2:*PhGATA18utr* or TRV2:*PhGATA18code* and TRV2:*CHS* positive plants using qRT-PCR on floral cDNAs as previously described (Hileman, Drea, Martino and Litt, 2005). Primers to test for gene silencing were selected based on their ability to anneal outside of the TRV2 insert region for *PhGATA18* and *PhGATA19* (Appendix 1 and 2).

RESULTS

***HAN*-like genes were duplicated at the base of Solanaceae**

Maximum likelihood and Bayesian topologies of *HAN*-like genes are largely congruent, and track the well-supported species phylogeny of asterids (Bremer et al., 2009). Both

analyses strongly support the hypothesis that Solanaceae species have at least two co-orthologs of *A. thaliana HAN* (and *HANL1* and *HANL2*) derived from a gene duplication event at the base of the Solanaceae (posterior probability (PP) of 1.0; 86% bootstrap support (BS)). According to previous publications, the resulting genes will hereafter be referred to as *GATA18* and *GATA19*. Although the three *A. thaliana HAN*-like genes fail to form a monophyletic clade as in previous analyses (Zhao et al., 2004; Behringer and Schwechheimer, 2015), their relative relationships are not well resolved, probably due to lack of sampling within the rosid clade of core eudicots. We also identified two *HAN*-like genes each from carnation and sugarbeet (*Beta vulgaris*) that appear to have evolved from a recent duplication event within the Caryophyllales.

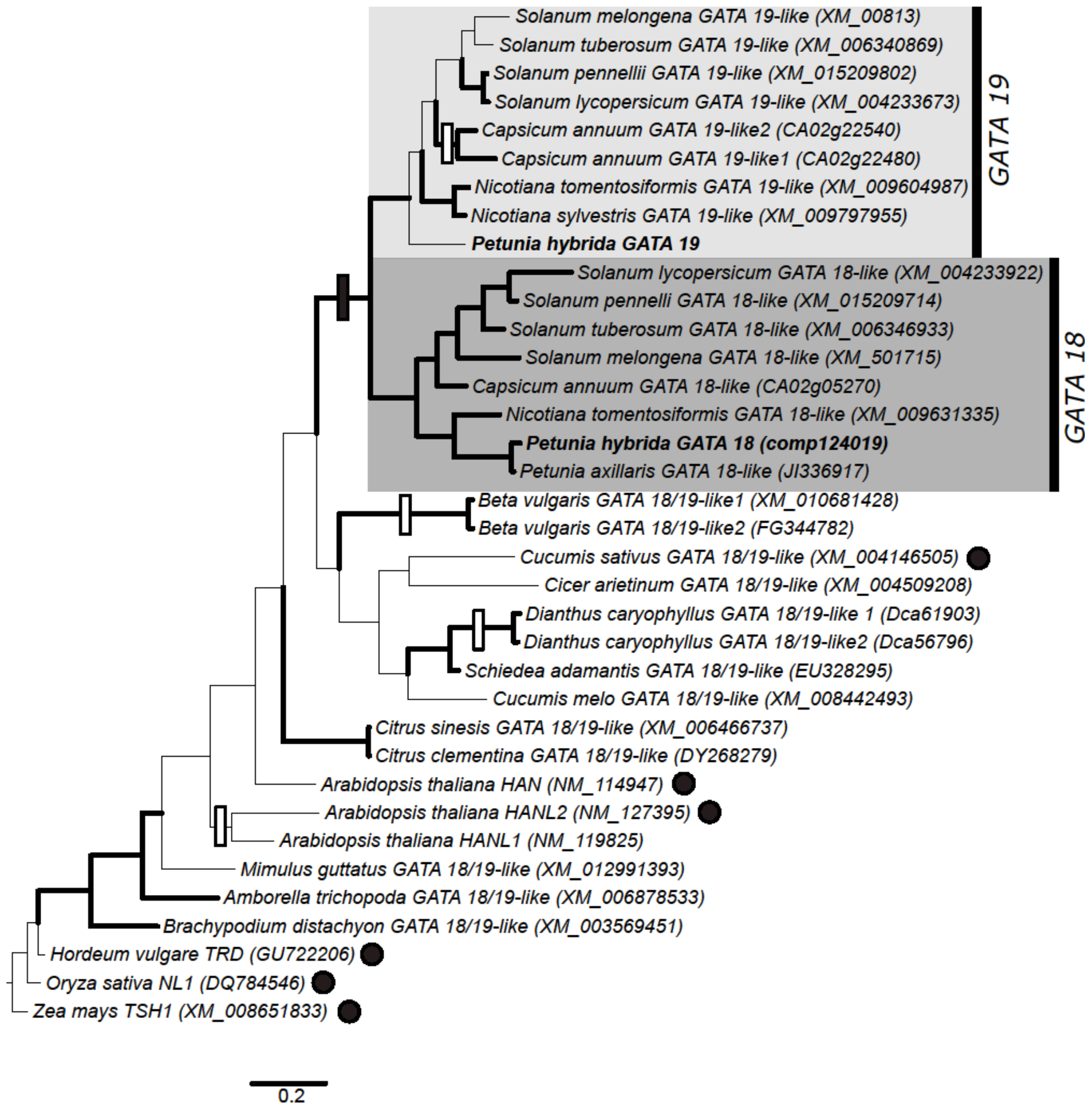

Figure 1. Bayesian inference of the phylogenetic relationships among *HAN*-like genes based on alignment of the conserved GATA domain and HAN motif. Duplication of *HAN*-like genes at the base of the Solanaceae is denoted by a solid black rectangle while other duplication events are denoted by open black rectangles. Highlighted sub-clades represent duplicated Solanaceae *HAN*-like genes. Focal species are highlighted in boldface text and genes previously characterized functionally are denoted by black circles. Thick branches indicate Bayesian posterior probabilities and maximum likelihood bootstrap values above 0.9 and 70%, respectively. Scale bar indicates substitutions per site.

HAN-like expression in wild-type carnation and petunia

In *A. thaliana*, *HAN* and *HANL2* are expressed in vegetative tissues, all floral organs, and particularly in the center of the floral meristem (Zhao et al., 2004; Winter et al., 2007; Zhang et al., 2013). To determine if *HAN* orthologs in carnation (Fig. 2A, top) and petunia (Fig. 2A, bottom) are similarly expressed, we conducted qRT-PCR analyses on different wild-type tissues (Fig. 2B-D, F and G). Expression of carnation *DcGATA18* is detectable in all above-ground tissues tested, with relatively high expression in floral versus vegetative tissues (Fig. 2B). Petunia *HAN*-like orthologs *PhGATA18* and *PhGATA19* are similarly broadly expressed across both vegetative and floral tissues (Figure 2C, D). However, whereas *PhGATA18* is most highly expressed in sepals of 10 mm flowers, *PhGATA19* is most highly expressed in the gynoecium (Fig. 2C, D). Unexpectedly, the petunia *HAN*-like orthologs also show an inverse expression pattern in dorsal compared to ventral petals, and across the length of the corolla. *PhGATA18* is expressed more strongly in the ventral relative to dorsal petals (Fig. 2A and C), and the lobe-transition zone-upper corolla tube region compared with the lower corolla tube (Fig. 2E and F). In contrast, *PhGATA19* expression is highest in the dorsal relative to ventral petals (Fig. 2A and D), and the lower corolla tube compared to the lobe-transition zone-upper corolla tube region (Fig 2E and G).

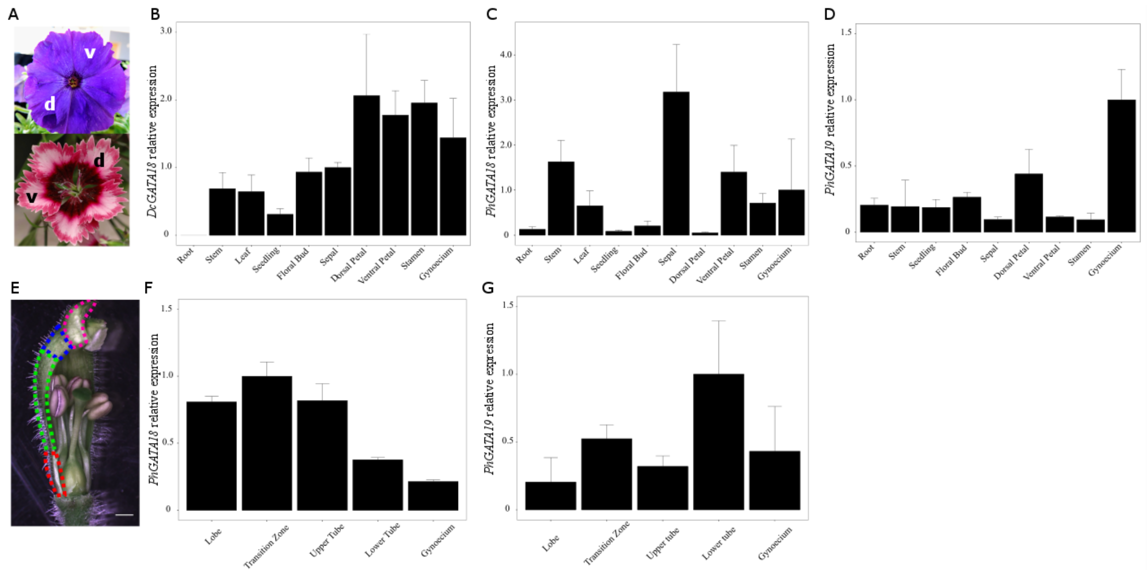

Figure 2. *HAN*-like gene expression patterns in carnation and petunia. (A) Wild-type petunia (top) and carnation (bottom) flowers showing resupinate dorsal (d) and ventral (v) regions. (B-D) Relative expression of carnation *DcGATA18* (B), petunia *PhGATA18* (C), and *PhGATA19* (D) across various wild type tissues. (E) Longitudinal section through a petunia flower showing lobes (pink), transition zone (blue), upper corolla tube (green), and lower corolla tube (red). (F,G) Relative expression of *PhGATA18* (F) and *PhGATA19* (G) in 10 mm long dissected floral buds. Transcript levels for three biological replicates ± SE were rescaled to expression levels of gynoecia.

Functional evolution of petunia *HAN*-like genes

To test the hypothesis that petunia *HAN*-like genes affect organ fusion, and more specifically regulate petal development, VIGS was conducted targeting *PhGATA18* and *PhGATA19*. The VIGS vector targeting the conserved *PhGATA18* GATA domain (TRV2:*PhGATA18code*), significantly reduced both *PhGATA18* and *PhGATA19* expression relative to control vector TRV2:*PhCHS* (Fig. 3A and B). By contrast, the TRV2:*PhGATA18utr* vector, which was constructed from the 3'-UTR region of *PhGATA18*, was more specific in its targeting, reducing the expression level of *PhGATA18*, but not *PhGATA19* (Fig. 3A and B).

Plants infected with TRV2:*PhGATA18code* or TRV2:*PhGATA18utr* showed no difference in the size, number of, or fusion among organs compared to plants infected with the control vector TRV2:*PhCHS* (Fig. 3C) or wild-type individuals (data not shown). However, dual silencing of *PhGATA18* and *PhGATA19* resulted in developmentally stunted plants with mottled leaves that senesced before transition to the flowering stage (Fig 3D). To determine whether stage of development at which plants were treated had an effect on survival, plants were infiltrated at the pre- and post-transition to flowering stages, which showed similar knockdown effects on *PhGATA18* and *PhGATA19* transcript levels as compared to plants infiltrated at the 4-6 leaf stage (Fig. 3A and B). Survival of plants infected with TRV2:*PhGATA18code* was 47% (n=31) compared to 100% survival in plants infected with TRV2:*PhCHS* (n=33), regardless of developmental stage at infiltration. The gene-specific construct TRV2:*PhGATA18utr* had no visible effect on vegetative development and survival (100%, n=27) compared to control plants (100%, n=25) (Fig. 3D).

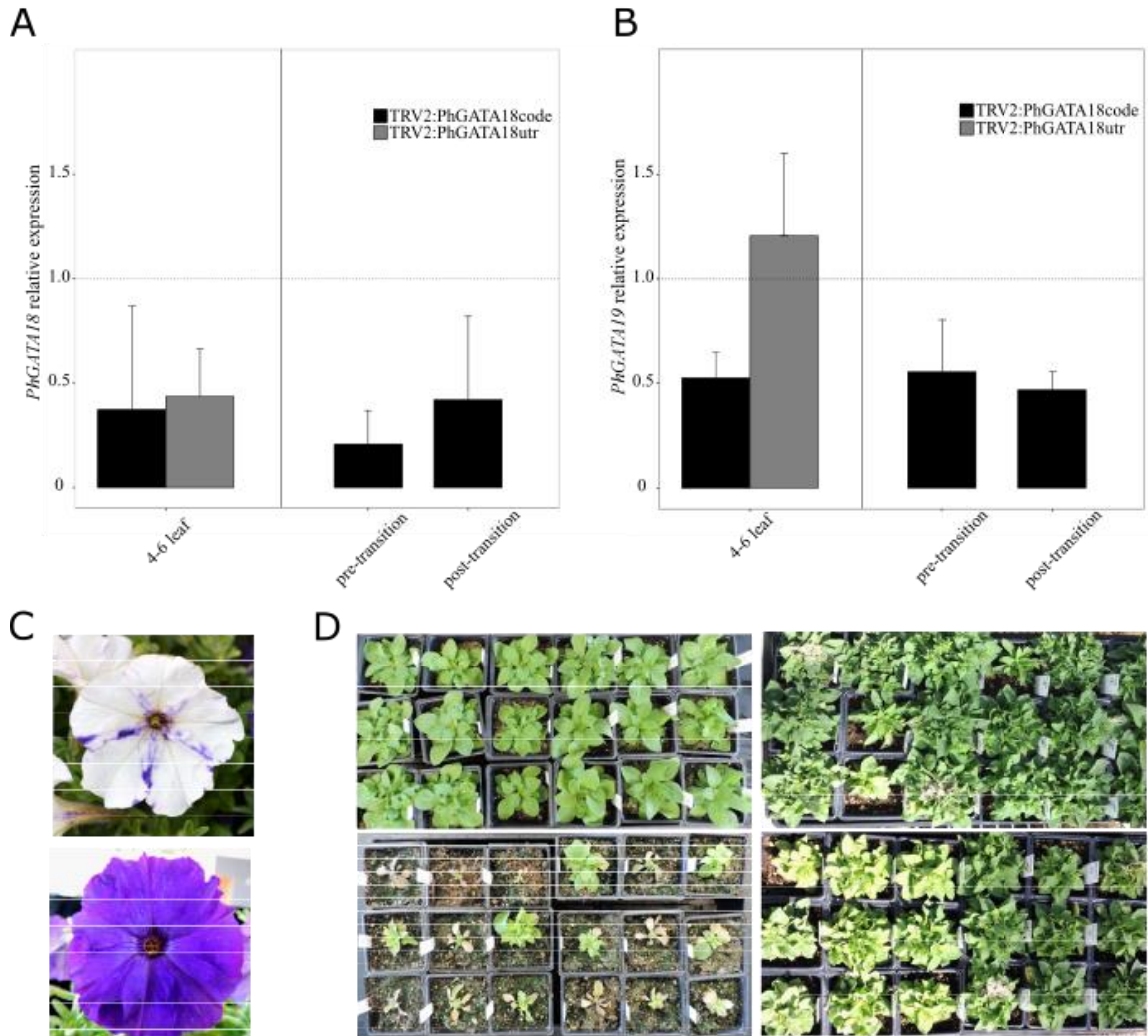

Figure 3. Virus-induced gene silencing of *PhGATA18* and *PhGATA19*. (A) *PhGATA18* transcript levels are significantly lower in *PhGATA18*code- and *PhGATA18*utr-TRV2- relative to *PhCHS*-TRV2-infected plants (dotted line), regardless of infection stage. (B) *PhGATA19* transcript levels are significantly lower in *PhGATA18*utr-, but not *PhGATA18*code-, TRV2- relative to *PhCHS*-TRV2-infected plants (dotted line). (C) Flowers of *PhCHS*-silenced plants (top) versus *PhGATA18code* knock-down plants (bottom) at 17 days post-infiltration. (D) Vegetative phenotype of *PhCHS*-silenced plants (top) versus *PhGATA18utr* knock-down plants (bottom) at 17 (left) and 33 (right) days post-infiltration. Transcript levels in (A) and (B) are based on at least five biological replicates ± SE; asterisks denote significant difference ($p<0.01$) from expression levels in *PhCHS* knockdown plants.

Discussion

Gene duplication, a common occurrence in plants, is an important source of genetic material upon which selection may act to produce phenotypic novelty. To this end, we asked whether retention followed by diversification of duplicated genes contributes to trait evolution in plants. Specifically, this study addressed the evolutionary direction and diversification of *HAN*-like genes and their contribution to shifts in petunia floral morphology. Data from this study suggest that petunia *HAN* paralogs have both a conserved and redundant function in promoting overall plant growth, while their involvement in petunia leaf and floral morphogenesis is still unclear.

Reconstruction of the evolutionary history of eudicot *HAN*-like genes supports the hypothesis that a gene duplication event occurred at the base of Solanaceae, giving rise to at least two co-orthologs of *A. thaliana HAN, HANL1,* and *HANL2*. Petunia *HAN*-like genes *GATA18* and *GATA19* show both distinct and overlapping patterns of expression in vegetative and floral tissues. Most notably, *PhGATA18* and *PhGATA19* show an inverse expression pattern in dorsal and ventral petals, and along the length of the corolla tube, suggesting that these paralogs may possess separate roles in petunia floral development while attenuating the ancestral function in SAM development.

The finding that simultaneously reducing expression of *PhGATA18* and *PhGATA19* results in stunted growth and early senescence suggests that these genes function redundantly in promoting overall plant growth, possibly through the control of hormone pathways and/or SAM regulation as demonstrated for *A. thaliana HAN* (Zhang et al., 2013). Combined with the similar phenotype found in cucumber, we infer that SAM regulation was the ancestral gene function at least in core eudicots. However, it is

not clear that *PhGATA18* and *PhGATA19* are involved in leaf or floral morphogenesis, as has been found in the rosid core eudicots, cucumber and *A. thaliana* (Zhao et al., 2004; Ding et al., 2015). The most obvious phenotype of *han* mutants in *A. thaliana* is reduction in petal number and fusion within the stamen and sepal whorls (Zhao et al., 2004), and in cucumber *han* perturbation affects leaf dissection (Ding et al., 2015). In contrast, no floral defects were observed in *PhGATA18* and *PhGATA19* knockdown plants, suggesting that petunia *HAN*-like genes are not involved in floral organ morphogenesis. In contrast, floral expression patterns of *PhGATA18* and *PhGATA19* indicate a divergence in function for these paralogs. A potential explanation for this incongruence between gene expression patterns and VIGS phenotypes is that petunia *HAN*-like genes need to be completely silenced to show all their effects, or that they are redundant with other genes affecting functions in floral organ morphogenesis.

Members of the *NO APICAL MERISTEM (NAM)-ARABIDOPSIS TRANSCRIPTION ACTIVATION (ATA) FACTOR-CUP-SHAPED COTYLEDON (CUC)* (*NAC*) and *WUSCHEL*-related *HOMEOBOX* (*WOX*) families have been implicated as regulators of boundaries between fused petunia petals (Souer et al., 1996; Vandenbussche et al., 2009; Segatto et al., 2013; Zhong et al., 2016). Silencing of petunia *NAM* results in several abnormal phenotypes such as fused sepals and stamens-carpels, and loss of fusion in petals (Zhong et al., 2016). Similarly, the petunia *WOX* transcription factor *MAEWEST* (*MAW*) is implicated in lateral outgrowth of petals, and thus petal fusion, as evidenced by unfused, thinner petals in *maw* mutant plants (Vandenbussche et al., 2009). Given that upregulation of *HAN* in *A. thaliana* alters expression of organ boundary genes *CUC3, RABBIT EARS* (*RBE*), and *JAGGED* (*JAG*), it would be interesting to see if petunia

HAN-like genes interact with these genes as well as with *MAW* and *NAM* in a similar pathway. It will also be important to determine whether a complete loss-of-function mutation in *PhGATA18* or *PhGATA19* leads to floral abnormalities as predicted by their patterns of expression.

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CHAPTER 3: SYNTHESIS AND FUTURE DIRECTIONS

1. SYNTHESIS

Results of my thesis support the occurrence of several *HANABA TARANU* (*HAN*) gene duplication events, including one at the base of the Solanaceae, giving rise to two co-orthologs (henceforth *PhGATA18* and *PhGATA19*) of *Arabidopsis thaliana HAN*, *HANL1*, and *HANL2* in petunia (*Petunia x hybrida*). By and large, expression patterns of *PhGATA18* and *PhGATA19* overlap in both vegetative and floral tissues, with the exception of an inverse pattern of expression in dorsal versus ventral petals and also along the length of the corolla tube. This inverse expression of petunia *HAN*-like genes implies independent function of each paralog in floral patterning, while their shared expression patterns suggest an attenuated ancestral function in vegetative and floral development. The observation that co-silencing of *PhGATA18* and *PhGATA19* causes impeded vegetative growth and premature leaf senescence further indicates that petunia *HAN*-like genes have a redundant function in the stimulation of plant growth, the specific mechanisms of which go beyond the scope of this particular study. However, based on evidence from *A. thaliana* (Zhang et al., 2013), I hypothesize that petunia *PhGATA18* and *PhGATA19* carry out their role in plant growth through regulation of the shoot apical meristem (SAM) in coordination with several hormone pathways.

Data to support this hypothesis include the widespread transcriptional repression in *A. thaliana HAN* overexpression lines of non-*HAN* GATA-3 transcription factors and genes integral to several hormone pathways, including auxin and cytokinin response as well as signaling in jasmonic acid and gibberellin pathways (Zhang et al., 2013). The

GATA-3 factors that *HAN* represses, *GATA NITRATE-INDUCIBLE CARBON-METABOLISM INVOLVED* (*GNC*) and *CYTOKININ-INDUCED GATA1/GNC*-like (*CGA1/GNL*, henceforth *GNL*), are involved in similar hormone actions (Naito et al., 2007; Richter et al., 2010) but also in nitrogen metabolism, glucose sensitivity, and chlorophyll biogenesis (Bi et al., 2005). Thus, my main working hypothesis is that petunia *PhGATA18* and *PhGATA19*, along with other GATA-3 transcription factors, regulate the fine-tuned cascade of senescence processes, including changes in hormone levels, chloroplast disassembly, nutrient redistribution and catabolism, and accumulation of reactive oxygen species (ROS) (Hörtensteiner and Feller, 2002; Hörtensteiner, 2006; Lim et al., 2007; Zwack and Rashotte, 2013; Munné-Bosch and Alegre, 2002). I further hypothesize that the early senescence phenotype in virus-induced gene silencing (VIGS) plants is a result of erroneous initiation of the plant's innate immune response to pathogen attack, thus shifting the plant's resources to programmed cell death in lieu of cell growth. If supported, this hypothesis would indicate that *PhGATA18* and *PhGATA19* may be operating as a "switch" of pathogen-mediated cell senescence, and without sufficient levels of these genes, the defense signaling cascade is launched more readily, even in the absence of a stimulus.

2. FUTURE DIRECTIONS

A suggested future experiment that would address the aforementioned hypotheses is the simultaneous and separate silencing of *PhGATA18* and *PhGATA19* by replacing VIGS with CRISPR/Cas genome editing technology followed by differential gene expression profiling of candidate downstream targets using RNASeq. CRISPR/Cas is a gene editing

approach that induces permanent small mutations in a specific gene or genes of interest through inefficient repair of double stranded breaks (Sander and Joung, 2014). Independent mutations yield stable allelic series that can range from silent or single amino acid changes to complete loss of function (knockout), as opposed to the transient post-transcriptional expression inhibition (knockdown) that is achieved using VIGS. The phenotypic outcome using a CRISPR/Cas system is therefore more varied and potentially much more robust than VIGS approaches to assess all facets of a gene's function.

Although a reference genome is currently unavailable, transcriptome datasets exist for *P. x hybrida* (Fernandez-Pozo et al., 2015). Additionally, sequenced and assembled parental genomes of *P. x hybrida* (*P. axillaris* and *P. inflata*) can be used as a reference for the *de novo* transcriptome assembly proposed here (Bombarely et al., 2016). Using these datasets, CRISPR/Cas systems can be designed to silence one and/or both petunia *HAN*-like genes. These CRISPR-edited plants can then be grown alongside wild-type petunia under standard greenhouse conditions and sampling of leaf tissue for RNA extraction can be done at regular intervals to capture the transcriptome through various stages of leaf development. *De novo* sequence assembly and prediction of coding regions can be reconstructed from RNASeq data using the Trinity platform (Haas et al., 2013) followed by gene ontology (GO) categorization (Ashburner et al., 2000). Differential expression analysis between control and mutant plants can then be carried out as previously described (Preston et al., 2016) to see if the transcription of GATA-3, hormone pathway genes, and immune response homologs is indeed affected by HAN-like gene mutations.

Upon completion, the results of such a study are expected to reveal the underlying functions of petunia *HAN*-like genes, and at the very least, such an experiment would highlight gene networks downstream of *PhGATA18* and *PhGATA19*. These data would not only help us understand growth and development in petunia, but also to determine to what extent senescence pathways are conserved across the eudicot clade of angiosperms.

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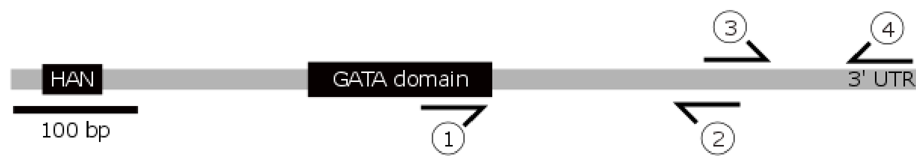
APPENDICES

Appendix 1. Primers used in this study

Primer	Sequence (5'-3')	Efficiency
gene isolation		
SolGATA19.F	TCTGCNTTCTCAAATGCTCTTCTCC	N/A
SolGATA19.R	CTAGGCCTATCAGCAACAYTG	N/A
Amplification of inserted sequences for VIGS constructs		
PhGATA18.utr.Bam.F	ACAGGATCCCATTCCCTTTTGGCCTTTC	N/A
PhGATA18.utr.Xho.R	ACACTCGAGTCCCTATTTTTGTTCTTCCTTAGC	
PhGATA18.code.Bam.F	ACAGGATCCTGCTTGTGGAATTCGTTTCA	N/A
PhGATA18.code.Xho.R	ACACTCGAGAGGCCAAAAGGGAATGCTAT	
PhGA19.B.476.F	ACAGGATCCGCAGTGGAGGAGTAGACACA	N/A
PhGA19.X.636.R	ACACTCGAGGCCAGCATCAGAATCACGTT	
PCR screening of infected plants		
PYL156F	GGTCAAGGTACGTAGTAGAG	N/A
PYL156R	CGAGAATGTCAATCTCGTAGG	
qRT-PCR		
DcEF1a.q.646.F	CTACTTGAGGCCCTTGACGA	98%
DcEF1a.q.747.R	CGTTCCAATACCACCGATCT	
DcHAN.q.F	AGGCTACTACAACGGGGGAT	107%
DcHAN.q.R	GTCCATGAACCGGAACTCGT	
DcUBQ5.q.48.F	GGTTGAATCCTCCGACACCA	85%
DcUBQ5.q.167.R	AGTGTACGGCCGTCTTCAAG	
PhEF1alpha.q.F	CCTGGTCAAATTGGAAACGG	104%
PhEF1alpha.q.R	CAGATCGCCTGTCAATCTTGG	
PhUBQ5.q.F	TGGAGGATGGAAGGACTTTGG	104%
PhUBQ5.q.R	CAGGACGACAACAAGCAACAG	
PhGATA18.q.a.F	GTGGAATTGGAGGAGGGAAT	107%
PhGATA18.q.g.R	CAATGCTGAGAAGCCATGAA	



Appendix 2. VIGS constructs and primer placement
1) PhGATA18code.Bam.F
2) PhGATA18code.Xho.R
3) PhGATA18utr.Bam.F
4) PhGATA18utr.Xho.R