Evolution of petaloid sepals independent of shifts in B-class MADS box gene expression

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ABSTRACT

Attractive petals are an integral component of animal-pollinated flowers, and in many flowering plant species are restricted to the second floral whorl. Interestingly, multiple times during angiosperm evolution, petaloid characteristics have expanded to adjacent floral whorls or to extra-floral organs. Here we investigate developmental characteristics of petaloid sepals in *Rhodochiton atrosanguineum*, a close relative of the model species *Antirrhinum majus* (snapdragon). We undertook this in two ways, first using scanning electron microscopy (SEM) we investigate micromorphology of petals and sepals, followed by expression studies of genes usually responsible for the formation of petaloid structures. From our data, we conclude that *R. atrosanguineum* petaloid sepals lack micromorphological characteristics of petals, and that petaloid sepals did not evolve through regulatory evolution of B-class MADS box genes, which have been shown to specify second whorl petal identity in a number of model flowering plant species including snapdragon. These data, in conjunction with other studies, suggests multiple convergent pathways for the evolution of showy sepals.

Key words: ABC model; *Antirrhinum majus* (snapdragon); B-class genes; petal identity; *Rhodochiton atrosanguineum*

INTRODUCTION

Flowers show a wide range of diversity, much of which can be attributed to evolutionary changes in the size, shape, number and color of petals or petaloid organs, which function to attract pollinators. It has been suggested that petals have been gained (De Craene 2007, Rasmussen et al. 2009, Soltis et al. 2009, Zanis et al. 2003) and lost (Jaramillo and Kramer 2007, Wu et al. 2007) multiple times during angiosperm evolution. Indeed, in some cases, such as, but not limited to, the genus *Cornus*, additional floral and extra-floral organs, including bracts, have evolved petaloid features (Brockington et al. 2009, Geuten et al. 2006, Maturen 2008, Rasmussen et al. 2009, Zhang et al. 2008).

The typical core eudicot flower consists of four concentric whorls of floral organs. The two outer-most organ whorls develop into a differentiated perianth surrounding the reproductive organs. In this typical flower, leaf-like sepals develop in the outer perianth whorl known as the calyx, and petals occupy the inner perianth whorl referred to as the corolla. Petals are often highly complex and are morphologically distinct from leaves and sepals

due to several characteristics, including the presence of colored pigmentation, conical or elongated epidermal cell shape, lack of stomata, and lack of palisade mesophyll (reviewed in De Craene 2008, Glover 2007, Irish 2009). Another characteristic that distinguishes petals from sepals and leaves is the necessary gene products for petal specification (reviewed in Irish 2009). Interestingly, many angiosperms develop flowers which do not conform to the typical form described above – most notably, those in which all perianth organs exhibit a petaloid appearance (*e.g.*, orchids, lilies, columbines, magnolias; reviewed in Kramer 2007, Litt and Kramer 2010), and the extent to which outer whorl perianth organs adopt petaloid characteristics has been studied in multiple taxa (Geuten et al. 2006, Kanno et al. 2003, Maturen 2008, Nakamura et al. 2005, Park et al. 2004).

For the typical flower with a differentiated perianth, the ABC model posits that the combination of three classes of genes, termed A, B, and C, function in overlapping domains to specify the formation of the different floral organ identities (Bowman et al. 1991, Coen and Meyerowitz 1991, Coen et al. 1991, Schwarz-Sommer et al. 1990, Trobner et al. 1992). Presence of A-class function determines sepals in the outer whorl, with co-occurrence of A- and B-class function determining petal identity in the second whorl. Stamens are determined in the third whorl by co-occurrence of B- and C-class function, and in the fourth whorl, occurrence of C-class function determines the identity of carpels. Therefore, based on the ABC model, B-class proteins play a critical developmental role in establishing petal identity, and in the differentiation of petal from sepal identity within the perianth.

B-class genes comprise two lineages: the *APETALA3*/*DEFICIENS* lineage (Jack et al. 1992, Sommer et al. 1990) and the *PISTILATA/GLOBOSA* lineage (Goto and Meyerowitz 1994, Trobner et al. 1992). Since their original characterization in *Arabidopsis thaliana* L. (Brassicaceae) and *Antirrhinum majus* L. (Plantaginaceae) (Bowman et al. 1989, Carpenter and Coen 1990, Krizek and Meyerowitz 1996, Schwarz-Sommer et al. 1990, Sommer et al. 1990) many additional studies have demonstrated that B-class function in establishing second whorl petal identity is conserved across angiosperms including flowering tobacco (*Nicotiana benthamiana* Domin, Solanaceae; Liu et al. 2004), tomato (*Solanum lycopersicum* L., Solanaceae; de Martino et al. 2006), petunia (*Petunia hybrida* Juss., Solanaceae; Rijpkema et al. 2006, Vandenbussche et al. 2004), poppy (*Papaver somniferum* L., Papaveraceae; Drea et al. 2007), and represents a conserved developmental pathway for lodicule specification in grasses (Kang et al. 1998, Ambrose et al. 2000, Prasad and Vijayraghavan 2003, Xiao et al. 2003, Whipple et al. 2004).

As mentioned above, many core eudicot species develop perianths that are undifferentiated or exhibit reduced differentiation, and therefore may not poses floral developmental programs that conform fully to the

canonical ABC model. To explain the developmental genetic program underlying undifferentiated perianths, the sliding boundary model was developed (Bowman 1997, Kramer et al. 2003). According to this model, A- and B-class function in both the inner and outer whorl perianth organs leads to an expansion of petal identity across the entire perianth. Data from multiple species, including tulip (*Tulipa gesneriana* L., Liliaceae; Kanno et al. 2003), lily (*Lilium longiflorum* Thunb., Liliaceae; Tzeng and Yang 2001), lily of the nile (*Agapanthus praecox* Willd., Alliaceae; Nakamura et al. 2005) and water lilies (*Cabomba caroliniana* Gray, Cabombaceae; Yoo et al. 2010) support this hypothesized genetic model as an explanation for the presence of an entirely petaloid perianth. However, many other species possess perianths that exhibit differentiation but with sepals that appear petaloid. For example, sepals may appear petaloid in shape, coloration or micromorphology, but not develop identically to second whorl petals. Examples of these flowers include columbine (*Aquilegia vulgaris* L., Ranunculaceae; Kramer et al. 2007), common heather (*Calluna vulgaris* (L.) Hull, Ericaceae; Borchert et al. 2009), gerbera (*Gerbera hybrida* L., Asteraceae; Broholm et al. 2010), orchids (*Habenaria radiata* (Thunb.) Spreng., Orchidaceae; Kim et al. 2007), impatiens (*Impatiens hawkeri* W. Bull, Balsaminaceae; Geuten et al. 2006), garden asparagus (*Asparagus officinalis* L., Asparagaceae; Park et al. 2003, Park et al. 2004), and members of the Aizoaceae (Brockington et al. 2011; Brockington et al. 2009). Within this diverse group, there does not appear to be a clear developmental genetic program explaining the formation of first whorl petaloid organs.

The focus of this study is *Rhodochiton atrosanguineum* L. (Plantaginaceae), a close relative of the model species snapdragon (Fig 1a). *Rhodochiton atrosanguineum* flowers do not appear to phenotypically adhere to the ABC model in the same fashion as snapdragon flowers with their distinct sepals and petals (Fig. 1c). *Rhodochiton atrosanguineum* flowers have outer and inner whorl perianth organs that are morphologically distinct, yet both whorls of organs exhibit a petaloid appearance (Fig 1b). In this study we aim to determine the extent to which outer whorl perianth organs of *R. atrosanguineum* exhibit petal identity beyond pigmentation of sepals. Therefore, we investigate perianth micromorphology with the specific hypothesis that *R. atrosanguineum* outer whorl perianth organs will exhibit characteristics of epidermal cell shape resembling inner whorl petals. Additionally, we test the applicability of the ABC genetic model, with the specific prediction that expression of B-class orthologs *DEF* and *GLO* will be detected in *R. atrosanguineum* outer whorl perianth organs if they have adopted petal identity.

METHODS

Plant material- Seeds of *R. atrosanguineum* were obtained from B and T World Seeds (http://www.b-and-t-world-seeds.com) and *A. majus* seeds, accession number ANTI 11 (D2836), were obtained from the Gatersleben collection (Leibniz Institute of Plant Genetics and Crop Research, http://www.ipk-gaterlseben.de). Voucher specimens of *R. atrosanguineum* (JL001 and JL002) and *A. majus* (JL004) have been placed in the R. L. McGregor Herbarium (KANU), University of Kansas. Flower material for both species was collected from plants grown in the greenhouse at the University of Kansas.

Scanning electron microscopy- Mature flowers of *A. majus* and *R. atrosanguineum* were fixed in glutaraldehyde (5% glutaraldehyde solution in 0.1 M phosphate buffer) overnight and then dehydrated through an ethanol series. Dehydrated flowers were critical point dried using a Tousimis critical point dryer and then dissected into sepals, base of petal tube, and petal lobe. Tissue was collected for imaging both adaxial and abaxial surfaces. Specimens were mounted on stubs, sputter-coated with gold, and viewed with a D. Leo field emission scanning electron microscope.

Isolation of *RaDEF* and *RaGLO* orthologs- Total RNA was isolated from immature *R. atrosanguineum* flowers using Tri-Reagent following the manufacturer instructions (Ambion, Austin, Texas, USA) and DNAse treated using TurboDNA (Ambion, Austin, Texas, USA). cDNA was generated using 1 µg of total RNA in a 15 µl cDNA synthesis reaction using iScript Synthesis Kit following the manufacturer instructions (BioRad, Hercules, California, USA). Orthologs of *DEF* and *GLO* were isolated from floral cDNA by reverse-transcriptase PCR (RT-PCR) using the degenerative forward primer (5'-AACAGGCARCTIACITAYTC-3') and the reverse PolyT-QT primer (5'-GACTCGAGTCGACATGGA$(T)_{18}$-3') (Hileman et al. 2006). This primer combination yields near-full length gene sequences, lacking only the 21 amino acids at the 5' end of the gene. RT-PCR reactions contained 2 ul of 1:10 diluted cDNA, 1.25 units *Taq* (Sigma-Aldrich, St. Louis, Missouri, USA), 10X PCR buffer, 0.5 µM of each primer, and 0.8 mM dNTPs. PCR reactions were run for 40 cycles with an annealing temperature of 47°C. PCR products were subjected to gel electrophoresis using a 1.5% agarose gel and gel-purified using the Wizard SV Gel and PCR Clean Up System Kit (Promega, Madison, Wisconsin, USA) before being cloned. Gel-purified PCR products were cloned into the pGEM -T vector system (Promega, Madison, Wisconsin, USA) following the manufacturer instructions. Twenty clones were sequenced using M13 forward and M13 reverse primers in order to identify multiple gene copies amplified by our RT-PCR approach (Howarth and Baum 2005).

Phylogenetic Analysis- Putative orthologs of *RaDEF* and *RaGLO* were aligned to additional B-class (*DEF/GLO*-like) genes downloaded from Genbank using MUSCLE (Edgar 2004), followed by manual adjustment in MacClade v4.08 (Maddison and Maddison 2005). Nucleotide sequence alignments were used to generate estimates of the gene phylogeny under Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian criteria. Maximum likelihood was implemented in Garli (Zwickl 2006) using the GTR + I + Γ model of molecular evolution. Support values using ML were generated with 1000 bootstrap replicates in Garli as described above. Support values were also generated using MP in Paup* 4.0 (Swofford 2002) with 1000 heuristic bootstrap replicates and the TBR branch swapping algorithm. Bayesian criterion was implemented using MrBayes (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003) and the GTR + I + Γ model of molecular evolution with two Markov chains running for 1,000,000 generations sampling every 100th generation. At completion of runs, the two chains were checked for convergence and the first 25% of saved trees were discarded as initial burn-in. Remaining trees were used to calculate posterior probabilities of node support in a 50% majority-rule consensus tree.

Expression of *RaDEF* and *RaGLO* by Reverse Transcriptase (RT)-PCR- *Rhodochiton atrosanguineum* RNA was extracted and cDNA generated from the four floral organ types of multiple flowers: outer-whorl petaloid sepals, petals, stamens and carpels, in three distinct floral size classes. The small size class included the earliest stage flower buds that could be hand-dissected, with corolla length in this size class ranging from 4.0 to 7.0 mm. Corolla length in the medium size class had a range of 15.0 to 18.0 mm. The large size class included flowers just pre-anthesis, with corolla length in this size class ranging from 39.5 to 40.5 mm. Expression patterns were characterized using gene specific primers designed to amplify fragments of *RaDEF* and *RaGLO* of ca. 150-200 bp of the open reading frame. Gene specific primers for amplifying *RaDEF* were *RaDEF-F (*5'-AGCTTGAACGATCTGGGCTA-3') and *RaDEF-R* (5'-GTGCGGATCCTCTCTTCTTG-3'), and primers for amplifying *RaGLO* were *RaGLO-F* (5'-GGGACGTCAGCTCTCAAAA-3') and *RaGLO-R* (5'-ATCGTATACCCCCTGGCTTT-3'). *ACTIN* was used a loading control as described by Prasad et al. (2001). RT-PCR reactions included 2 μl of 1:10 diluted cDNA, 1.25 units *Taq* (Sigma-Aldrich, St. Louis, Missouri, USA), 10X PCR buffer, 0.5 μM of each primer, and 0.8 mM dNTPs. PCR conditions consisted of 26 cycles with an annealing temperature of 55°C for all genes tested. The number of cycles was determined as that representing the linear range of amplification from a PCR product curve including reactions run for 22-40 cycles. Triplicate RT-PCR reactions for each cDNA, including RT-PCR negative control cDNAs (-RT), were conducted to ensure consistency.

Expression of RaDEF and RaGLO by in situ mRNA hybridization- Flower buds of *R. atrosanguineum* were fixed in FAA (47.5% ethanol, 5% acetic acid, 3.7% formaldehyde) for 8 hours, stained with eosin Y, dehydrated and wax embedded as described by Jackson (1991) and Preston and Kellogg (2007). Gene specific probe templates of *RaDEF* and *RaGLO* were generated using primers *RaDEF-F* (5'-AATACATCAGTCCCACCACAGC-3'), *RaDEF-R* (5'-GCAAAGCAAATGTGGTAAGGTC-3'), *RaGLO-F* (5'-TCATCATCTTTGCTAGTTCTG-3'), and *RaGLO-R* (5'-TCCTGAAGATTAGGCTGCATTG-3'). Probes were 520 bp and 488 bp long for *RaDEF* and *RaGLO* respectively, with forward primers in the I domain and reverse primers in the C-terminal coding region of each gene (Yang et al. 2003), to exclude amplification of the highly conserved MADS domain. All PCR products for probe generation were cloned into the pGEM -T vector (Promega, Madison, Wisconsin, USA) and confirmed by sequencing. Sense and antisense riboprobes for *RaDEF* and *RaGLO* were generated using T7 and SP6 RNA polymerase (Roche, Indianapolis, Indiana, USA) incorporating digoxygenin dNTPs (Roche, Indianapolis, Indiana, USA) according to the manufacturers instructions. Probe hydrolysis followed Jackson (1991) to yield fragments c. 150 bp long. In situ hybridization was performed on longitudinal sections of multiple inflorescences as in Jackson (1991). Multiple floral sections hybridized with antisense or sense probe were visualized to ensure consistent assessment of gene expression. Images were documented using a Leica DM5000B microscope attached to a Leica DFC300FX camera. Images were imported into Adobe Photoshop and adjusted for contrast, brightness and color balance.

RESULTS

Micromorphological analysis of petaloid sepals- To determine whether *R. atrosanguineum* petaloid sepals exhibit micromorphological cell shape characteristics found in *R. atrosanguineum* petals, SEM analyses were undertaken. Figure 2 shows image comparisons of epidermal cell shape from both the abaxial and adaxial surface of snapdragon (Fig. 2a-i) and *R. atrosanguineum* (Fig. 2j-r) leaves, sepals or petaloid sepals, and petals. Leaves (Fig. 2a,e,j,n), sepals and petaloid sepals (Fig. 2b,f,k,o) of both species show a consistent jigsaw-shaped cellular pattern on both the abaxial and adaxial surfaces, with stomata found predominantly on the abaxial surface of these organs (Fig. 2a,b,j,k). Jigsaw-shaped cells are also observed on the abaxial surface of both snapdragon and *R. atrosanguineum* petal lobes (Fig. 2d, m); interestingly, stomata are also found on the abaxial surface of *R. atrosanguineum* petal lobes (Fig. 2m), but not the abaxial surface of snapdragon petal lobes (Fig. 2d). *Rhodochiton atrosanguineum* abaxial petal lobe epidermal cells not only develop stomata but also appear more domed, or

lenticular, than corresponding snapdragon epidermal cells (Fig. 2d,m). In both species, elongated cells were found on both adaxial and abaxial surfaces at the base of the corolla tube (Fig. 2c,g,l,p). The major difference between the two species is found on the surface of adaxial petal lobes. The adaxial surface of *A. majus* petal lobes (Fig. 2h,i) exhibit papillose conical cells as previously documented (Noda et al. 1994, Perez-Rodriguez et al. 2005), while the adaxial petal lobes of *R. atrosanguineum* (Fig 2q,r) lack conical cells. *Rhodochiton atrosanguineum* adaxial petal lobe epidermal cells are more domed than the papillose conical epidermal cells found in snapdragon, and are referred to as lenticular (Kay et al. 1981). SEM images show no distinct micromorphological differences between petaloid sepals and leaf like sepals of *R. atrosanguineum* and snapdragon, respectively, and *R. atrosanguineum* petaloid sepals do not resemble *R. atrosanguineum* petals at the micromorphological scale.

Isolation and phylogenetic assessment of *RaDEF* and *RaGLO*- Two B-class genes were isolated from floral cDNA of *R. atrosanguineum* and deposited in GenBank (*RaDEF* JQ173625*; RaGLO* JQ173626). ML, MP and Bayesian phylogenetic estimates place one of these two genes (*RaDEF*) in a well-supported clade with *A. majus DEF*, and the other gene (*RaGLO*) in a clade with *A. majus GLO* (Fig. 3). *RaDEF* is nested within a clade of orthologs from snapdragon and *Misopates orontium* (L.) Raf. (Plantaginaceae), all of which are members of the tribe Antirrhineae. This clade has bootstrap support values of 97% (MP) and 98% (ML), and a posterior probability of 1.0 (Fig. 3). The placement of *RaGLO* is also in a well-supported clade with *GLO*-like genes from snapdragon and *M. orontium* with bootstrap values of 100% (MP) and 90% (ML) and a posterior probability of 1.0 (Fig. 3). The sister relationship between snapdragon and *M. orontium* is highly supported and reflects species relationships based on other molecular markers (Vargas et al. 2004).

Expression of *RaDEF* and *RaGLO*- Scoring of RT-PCR gene expression was dichotomous – presence or absence of *RaDEF* or *RaGLO* transcripts in sampled tissues. *RaDEF* and *RaGLO* transcripts were detected by RT-PCR in petals and stamens, but not in petaloid sepals or carpels, for the three size classes of flowers (Fig. 4). In addition, *RaDEF* (Fig. 5a) and *RaGLO* (Fig. 5c) expression was detected in developing petals and stamens, but not developing sepals of early-stage *R. atrosanguineum* flowers by *in situ* mRNA hybridization. These results demonstrate that from early through late stages of flower development, the B-class genes, *RaDEF* and *RaGLO*, are not expressed in *R. atrosanguineum* petaloid sepals.

Comment [JL1]: Overly repetitive information regarding the results of gene expression was removed.

DISCUSSION

Rhodochiton atrosanguineum sepals are brightly pigmented giving them a superficial petaloid appearance (Fig. 1b). The objective of this study was to determine to what extent *R. atrosanguineum* petaloid sepals have adopted petal characteristics at the micromorphological and molecular level. Specifically, we used SEM and gene expression studies to test the following hypotheses: 1) *R. atrosanguineum* petaloid sepals exhibit micromorphological characteristics found in adjacent petals, and 2) petaloid sepal development in *R. atrosanguineum* is associated with B-class MADS box gene regulatory evolution. We found that petaloid sepals of *R. atrosanguineum* do not morphologically resemble the petals of *R. atrosanguineum*, similar to studies in *Impatiens* (Geuten et al. 2006). Additionally, we found that the ABC genetic model, specifically expression of B-class genes restricted to the second and third flower whorls, is conserved in *R. atrosanguineum* despite development of petaloid sepals.

If, in *R. atrosanguineum*, the sepals have evolved a petaloid appearance due to expansion of petal identity to the first floral whorl, then we expect epidermal cell shape in these outer whorl organs to resemble epidermal cell shapes found in the second whorl petals. *Rhodochiton atrosanguineum* petaloid sepals develop jigsaw shaped cells, very similar to the cells found in sepals of snapdragon (Figs. 2b and 2k). These jigsaw shaped cells are distinct from the adaxial epidermal cells of *R. atrosanguineum* petals (Figs. 2h,i and 2q,r), which are dome-shaped and distinguish second whorl petals at the micromorphological level from *R. atrosanguineum* petaloid sepals and leaves. It is noteworthy that conical or papillose cell shape is found on the adaxial petal surface of many flowering plant species (Christensen and Hansen 1998, De Craene 2008, Kay et al. 1981, Whitney and Glover 2007), and these predicted papillose cells were found on the adaxial epidermis of snapdragon petals as previously described (Noda et al. 1994, Perez-Rodriguez et al. 2005) (Fig. 2h and i). Cell micromorphology of *R. atrosanguineum* petal lobes differs from snapdragon – lacking true papillose cells shape (Figs. 2h and 2p). The ultimate cause of micromorphological differences between *R. atrosanguineum* and snapdragon petals is unknown, but may reflect evolutionary shifts in biotic pollination mechanisms (Cronk and Ojeda 2008, Di Stilio et al. 2009); snapdragon is bee pollinated (Glover and Martin 1998, Whitney et al. 2009) while *R. atrosanguineum* is pollinated by hummingbirds (Sutton 1988).

A single copy of *DEF* and *GLO* were isolated from *R. atrosanguineum* and, based on our phylogenetic estimates (Fig. 3), are orthologous to *DEF* and *GLO* from snapdragon, respectively. The single copy of *RaDEF* and *RaGLO* are likely the only B-class homologs in *R. atrosanguineum*. The degenerative forward primer that was used to isolate these genes was situated in the MADS domain which is highly conserved (Yang et al. 2003), and the

Comment [JL2]: Information about cell shape was condensed to make the discussion more concise.

primer was designed to encompass variation in *DEF* and *GLO* across multiple Antirrhineae sequences. A combination of RT-PCR and *in situ* mRNA hybridization analyses demonstrate that both *RaDEF* and *RaGLO* expression is restricted to the petals and stamens from early to late-stages of *R. atrosanguineum* flower development (Figs. 4 and 5). Therefore, expansion of B-class gene expression to outer whorl perianth organs is not responsible for the petaloid appearance of *R. atrosanguineum* sepals. Interestingly, other studies have implicated another MADS box protein, *SEP3*, in conjunction with either *DEF* or *GLO*, in the evolution of a petaloid first whorl floral organs (Geuten et al. 2006). Because neither *RaDEF* nor *RaGLO* are expressed in the petaloid sepals, a model invoking the combined action of RaSEP with either RaDEF or RaGLO protein in first whorl organs can be rejected. Although *R. atrosanguinem* outer whorl organs are petaloid in appearance, primarily due to coloration, they are morphologically quite distinct from the inner whorl petals (Fig. 1b and 4) suggesting that changes in the anthocyanin pathway alone (Weiss 2000, Whittall et al. 2006) may underlie the evolution of petal-like sepals.

This study joins other studies that together indicate there is not a single developmental genetic model that explains the evolution of petaloid sepals (Borchert et al. 2009, Brockington 2009, Broholm et al. 2010, Geuten et al. 2006, Kim et al. 2007, Kramer et al., 2007, Park et al. 2004). Clearly convergent mechanisms lead to the development of petaloid sepals, and may involve evolutionary changes at the level of B-class gene expression, upstream or downstream of B-class genes, or parallel pathways (Jaramillo and Kramer 2004, Kramer et al. 2007, Litt and Kramer 2010). Interestingly *R. atrosanguineum* develops a differentiated perianth (Fig. 1b) common to core eudicots, lacks duplicates of *DEF* and *GLO*, and gene expression data from this species does not support the expansion of B-class genes to the outer perianth whorl. Because B-class genes play a critical role in establishing a bipartite perianth, constraints are likely present for the evolution of petaloid sepals by mechanisms involving B-class gene regulation (Hileman and Irish 2009, Kramer et al. 2003). Strikingly, when expansion of B-class gene expression is associated with the evolution of petaloid outer whorl perianth organs, it is restricted to species that have a history of gene duplication in *DEF* and/or *GLO* lineages, or species lacking a differentiated perianth (Kanno et al. 2003, Litt and Kramer 2010, Nakamura et al. 2005, Sharma et al. 2011, Tzeng and Yang 2001, Yoo et al. 2010). This study provides support for the emerging pattern that there are multiple, convergent developmental genetic mechanisms underlying independent transitions to petaloidy in the outer whorl perianth, and that constraint lies, at least in part, in whether there is morphological differentiation within the perianth, and whether there is a history of duplication in the B-class gene lineages.

Comment [JL3]: Information was removed to shorten the discussion, as well as address comments from reviewers about SEP3 and the inclusion of anthocyanin pathway.

Comment [JL4]: Kramer references removed from this section in response to reviewer's comments

FIGURE CAPTIONS

Fig. 1 Phylogenetic context of study species. a) ITS phylogeny adapted from Vargas et al. (2004) with new sequences added, showing relationships within the tribe Antirrhineae (Plantaginaceae). Focal genera *Rhodochiton* and *Antirrhinum* are bold faced to exemplify their relationship to each other. b) *Rhodochiton atrosanguineum* with petaloid sepals, c) *Antirrhinum majus* (snapdragon) with showy pink petals and leaf-like sepals

Fig. 2 Scanning electron microscope (SEM) images from *Antirrhinum majus* (snapdragon; a-i) and *Rhodochiton atrosanguineum* (j-r). Individual SEM images of *A. majus* are a) abaxial leaf, b) abaxial sepal, c) abaxial base of petal tube, d) abaxial petal lobe, e) adaxial leaf, f) adaxial sepal, g) adaxial base of petal tube, h) adaxial petal lobe top down, i) adaxial petal lobe 60° angled view. Individual SEM images of *R. atrosanguineum* are j) abaxial leaf, k) abaxial sepal, l) abaxial base of petal tube, m) abaxial petal lobe, n) adaxial leaf, o) adaxial sepal, p) adaxial base of petal tube, q) adaxial petal lobe, r) adaxial petal lobe 60° angled view. Images show conserved jig-saw shaped patterns of cell shape in leaves and sepals of the two species, and conserved elongated tubular cells at the base of the petal tube between both species. *Rhodochiton atrosanguineum* lacks conical cells on the adaxial surface of the petal lobe as seen in snapdragon, where as the abaxial petal lobe of *R. atrosanguineum* has a more defined cell shape than those seen in snapdragon. Scale bars in c, g, p are 30 µm, scale bars in h, i, q are 10 µm, all others are 20 µm

Fig. 3 Phylogeny of *DEFICIENS* (*DEF*) and *GLOBOSA* (*GLO*) orthologs showing placement of newly sequenced *Rhodochiton atrosanguineum DEF* and *GLO*. Shown is the maximum-likelihood (ML) tree with support values from 1000 replicate Maximum Parsimony (MP) bootstrap analysis/1000 replicate ML bootstrap analysis/Bayesian posterior probabilities. Only support values of >75% for bootstrap and >0.95 Bayesian posterior probabilities are shown on the phylogeny. The following are GenBank accession numbers for taxa other than *R. atrosanguineum*: ***Antirrhinum majus*** L.**;** AmDEF X52023, AmGLO AB516403. ***Arabidopsis thaliana*** L.; AtAP3 NM_115294, AtPI NM_122031. ***Brassica napus*** L.; BnAP3 DQ372719. ***Camellia japonica*** L.; GQ141126. ***Chelone glabra*** L.; CgDEF AY524008. ***Diospyros digyna*** Jacq.; DdGLO GQ141136. ***Lycopersicon esculentum*** L.; LeTAP3 DQ674532, LeTPI DQ674531. ***Mimulus guttatus*** DC.; MgDEFA AY524012, MgDEFB AY524020. ***Mimulus***

kelloggi (Curran ex Greene) Curran ex A. Gray; MkDEF AY530545. ***Misopates orontium*** (L.) Raf.; MoDEF AM162207, MoGLO Am162211. ***Napoleona vogelii*** Hook. & Planch; NvGLO GQ141117. ***Papaver somniferum*** L.; PsAP3-1 EF071993, PsAP3-2 EF071992, PsPI-1 EF071994, PsPI-2 EF071995. ***Paulownia tomentosa*** (Thunb.) Steud.; PtDEF AY524018. ***Petunia hybrida*** Juss.; PhDEF DQ539416. ***Phlox paniculata*** L.; PpDEF GQ141172, GQ141129. ***Saxifraga caryana*** L.; ScAP3 DQ479367. ***Syringa vulgaris*** L.; SvAP3 DQ479367, SvPI-1 AF052861. ***Torenia fournieri*** L.; TfGLO AB359952

Fig. 4 RT-PCR conducted on cDNA generated from three size classes of *R. atrosanguineum* flowers separated into their four floral organs. Small class flowers had a corolla length ranging from 4-7 mm, medium class flowers had a corolla length ranging from 15-18 mm, with large class flowers having corolla lengths of 39.5-40.5 mm. For all size classes, *DEFICIENS* and *GLOBOSA* were only expressed in the petals and stamens as seen by presence of bands in these organs. *ACTIN* was used as a loading control during RT-PCR analysis to insure the integrity cDNA. -RT samples served as negative controls, and no bands were visible in these reactions. *ACTIN* was also tested to show integrity of cDNA samples. *ACTIN* was expressed in all cDNA samples but in none of the -RT samples. Petaloid sepals = sep, petals = pet, stamens = sta, and carpels = car

Fig. 5 *In situ* hybridization conducted on early stage flower buds of *R. atrosanguineum.* a) Expression of *RaDEF* using antisense probe. b) Sense probe control for *RaDEF*. c) Expression of *RaGLO* using antisense probe. d) Sense probe control for *RaGLO.* Dark blue staining using antisense probes for *RaDEF* and *RaGLO* show that expression of these genes is limited to the developing petals and stamens of *R. atrosanguineum*. Petaloid sepals = sep, petals = pet, stamens = sta, and carpels = car

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