FUNCTIONAL EVOLUTION OF THE *APETALA1/FRUITFULL* GENE LINEAGE

by

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ABSTRACT

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Several MADS-box gene lineages involved in flower development have undergone duplications that correlate with the diversification of large groups of flowering plants. In the *APETALA1* gene lineage, a major duplication coincides with the origin of the core eudicots, resulting in the *euFUL* and the *euAP1* clades. Arabidopsis *FRUITFULL* (*FUL*) and *APETALA1* (*AP1*) function redundantly in specifying floral meristem identity, but function independently in sepal and petal identity (*AP1*) and in proper fruit development and determinacy (*FUL*). Many of these functions are largely conserved in other core-eudicot *euAP1* and *euFUL* genes, but notably the role of *APETALA1* as an "A-function" (sepal and petal identity) gene is thought to be Brassicaceae-specific. Understanding how functional divergence of the core-eudicot duplicates occurred requires a careful examination of the function of pre-duplication (*FUL-like*) genes. Using Virus Induced Gene Silencing (VIGS), it is shown that *FUL-like* genes in *Papaver somniferum* (opium poppy) and *Eschscholzia californica* (California poppy) function in axillary meristem growth and in floral meristem and sepal identity, and play a key role in fruit development. Interestingly, in opium poppy, these genes also control flowering time and petal identity, suggesting that *AP1/FUL* homologs might have been independently recruited in petal organ identity. In contrast,

it is shown that the *Aquilegia coerulea FUL-like* homolog does not appear to play a role in flower or fruit development and instead has been recruited in leaf morphogenesis. In general the *FUL-like* gene functional repertoire encompasses all roles previously described for the core-eudicot *euAP1* and *euFUL* genes, and subfunctionalization can be postulated as the functional outcome after the major *AP1/FUL* gene lineage duplication event. However, these results also point to significant functional variability of *FUL-like* genes within Ranunculales, most likely due to gene duplication and loss, as well as changes of FUL-like protein partners in different taxa.

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CHAPTER ONE

THE *APETALA1/FRUITFULL* GENE LINEAGE

1.1. MADS box genes: developmental regulators in floral development

MADS-box genes are transcription factors involved in a wide variety of developmental processes in eukaryotes (Becker and Theissen, 2003). In angiosperms, the sequential onset of expression of these transcription factors is responsible for proper flower development among other processes (Coen and Meyerowitz, 1991; Pelaz et al., 2000; Becker and Theissen, 2003). The ABC model (Fig. 1.1), based on the model species *Arabidopsis thaliana* (Brassicaceae) and *Antirrhinum majus* (Plantaginaceae), describes the specification of the identity of floral organs as a result of the function of A-class genes (in *Arabidopsis, AP1* and *AP2*), involved in sepal and petal identity, B-class genes (in *Arabidopsis, AP3* and *PI*), controlling petal and stamen identity, and C-class genes (in *Arabidopsis, AG*), responsible for the identity of stamens and carpels (Bowman et al., 1991; Coen and Meyerowitz, 1991; Bowman et al., 1993). According to the model, A and C class genes are mutually exclusive and actively repress each other in the floral meristem (Fig. 1.1) (Causier et al., 2010). Additional MADS-box genes have been found to be pivotal in floral meristem determinacy and to be required for the successful initiation of all floral organs, which has resulted in the inclusion of the E-class genes (*SEP1, SEP2, SEP3, SEP4*) in the model (Pelaz et al., 2000; Pelaz et al., 2001).

A subset of MADS-box proteins in plants, referred to as the MIKCc proteins, typically have four regions (Fig. 1.2) (Krizek and Meyerowitz, 1996; Kaufmann et al., 2005); a highly conserved MADS domain that encodes a region of approximately 60 amino acids important for DNA binding (Schwarz-Sommer et al., 1992; Riechmann et al., 1996; West et al., 1998; West and Shamrocks, 1999; Melzer et al., 2009), an I (Intervening) domain of variable length, a K (Keratin-like) domain, found to be important for protein dimerization, (Davies et al., 1996; Egea-Cortines et al., 1999; Yang et al., 2003; Yang and Jack, 2004), and a C-terminal domain highly variable in sequence, with small conserved motifs characteristic of different MADS-box gene lineages (Litt and Irish, 2003; Vandenbussche et al., 2003; Kramer et al., 2004; Zahn L.M., 2005; Kramer et al., 2006). Functions localized to the C-terminus include transcription activation, higher order complex formation and protein modification, but the role of most specific motifs is unknown (Cho et al., 1999; Egea-Cortines et al., 1999; Yalovsky et al., 2000). MADS box proteins are thought to form multimeric complexes in order to perform their organ identity function during flower development (Honma and Goto, 2001; Theissen, 2001). Specific combinations of four A, B, C and E proteins are formed in different domains of the meristem resulting in sepal, petal, stamen and carpel identity (Kaufmann et al., 2005; Melzer and Theissen, 2009; Melzer et al., 2009).

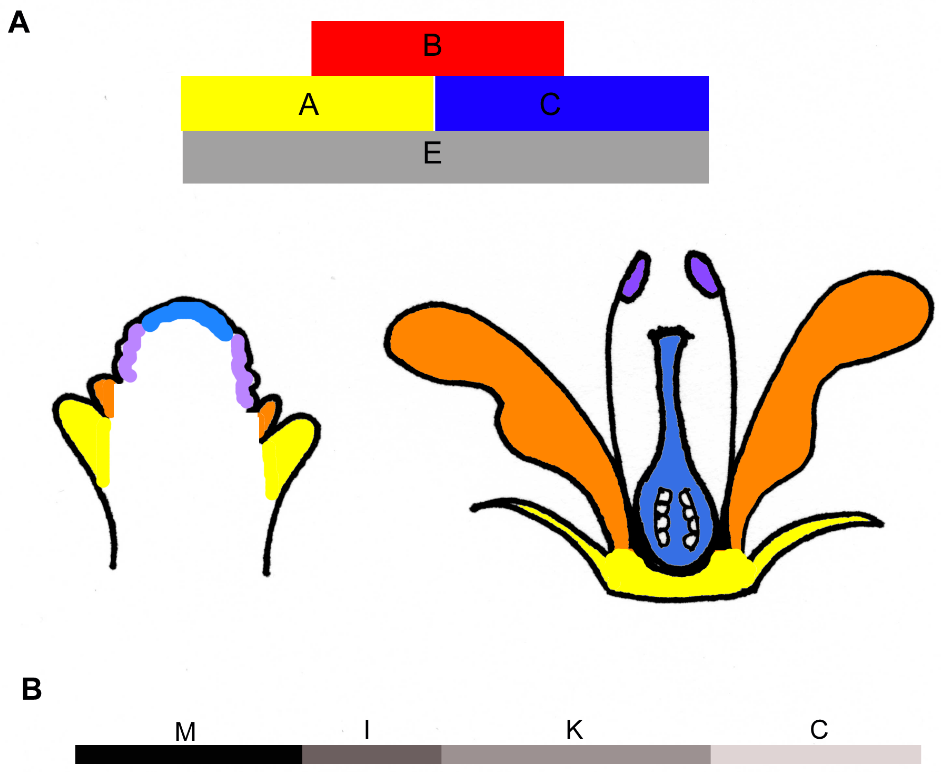

Figure 1.1. Schematic diagram of the ABC model.

A. The A, B and C class genes are responsible for the identity of the floral organs from the floral meristem as follows: A class genes control sepal and petal identity (perianth), B class genes control petal and stamen identity, and C class genes control stamens and carpels (reproductive organs). E class genes are involved in proper identity of all floral organs (Coen and Meyerowitz, 1991). Sepals are shown in yellow, petals in orange, stamens in purple and carpel in blue. Sections show in the left a floral meristem with sepal, petal, stamen and carpel primordia and in the right a mature flower. **B.** Map of a MADS box MIKC protein structure showing the four typical domains.

This model explains for the most part the genetic basis for floral development in *Arabidopsis* (Coen and Meyerowitz, 1991; Honma and Goto, 2001; Melzer and Theissen, 2009; Melzer et al., 2009), but is continuously being revisited in other angiosperms (Kramer et al., 1998; Kramer and Irish, 2000; Kim et al., 2004; Kim et al., 2005; Adam et al., 2007; Wu et al., 2007; Leseberg et al., 2008; Causier et al., 2010) in which number of copies, expression patterns and function of A, B and C class genes do not exactly match the ones outlined by the model. Variation in the number of gene copies in different plant groups is largely attributed to gene duplication and loss events (Ohno, 1970; Moore and Purugganan, 2005; Flagel and Wendel, 2009). Gene duplication is thought to provide raw material for functional diversification in gene lineages (Ohno, 1970; Lynch and Conery, 2000; Lynch and Force, 2000; Lynch, 2007). According to the theory, after duplication, gene copies can become pseudogenes (non functional copies often highly divergent in sequence), can perform the same function (redundancy), can divide the ancestral function (subfunctionalization) or can perform new, perhaps unrelated different functions (neofunctionalization) (Lawton-Rauh et al., 1999; Rastogi and Liberles, 2005; Roth et al., 2007; Flagel and Wendel, 2009). Gene copies can vary in terms of sequence and regulation. Likewise, protein structure and protein interaction can change, potentially resulting in phenotypic changes.

Gene duplication in several MADS-box gene lineages has occurred in different plant groups, and it has been particularly well documented in flowering plants (Purugganan, 1997; Alvarez-Buylla et al., 2000; Becker and Theissen, 2003). A major duplication event in the *APETALA1* (*AP1*), *APETALA 3* (*AP3*), *AGAMOUS* (*AG*) and *SEPALLATA* (*SEP*) gene lineages coincides with the origin of the core eudicots (Kramer et al., 1998; Litt and Irish, 2003; Kim et al., 2004; Kramer et al., 2004; Zahn L.M., 2005). After the duplication in the *AP1* and the *AP3* lineages, copies diverged in terms of sequence, attributed to a frameshift in the C terminal domain (Litt and Irish, 2003; Vandenbussche et al., 2003; Kramer et al., 2006). Comparative functional and expression studies of *AP3* and *AG* lineage members in monocots, basal eudicots and core eudicots suggest that, in general, the functions of these genes in petal, stamen and carpel identity (B and C function) are conserved before and after the core-eudicot duplication (Ambrose et al., 2000;

Jaramillo and Kramer, 2004; Gómez-Mena et al., 2005; Kim et al., 2005; De Martino G., 2006; Drea et al., 2007; Kramer et al., 2007; Di Stilio et al., 2010; Yellina et al., 2010; Dreni et al., 2011; Sharma et al., 2011). However, available data are lacking with which to evaluate the effect of the core eudicots duplication on the function and evolution of the *APETALA1* lineage; furthermore, the data that do exist have led to doubts about the conservation of A-function (Litt and Irish, 2003; Zik and Irish, 2003; Litt, 2007; Causier et al., 2010). In particular, data are lacking from basal eudicot species that would allow the evaluation of functional consequences as a result of the *AP1/FUL* duplication event in core eudicots.

1.2. The *AP1/FUL* gene lineage: duplication events and sequence divergence

The *AP1/FUL* gene lineage is exclusive to angiosperms and has undergone numerous duplication events in different angiosperm clades (Figure 1.2).The duplication event that coincided with the origin of the core eudicots resulted in the eu*FUL* and eu*AP1* clades (Litt and Irish, 2003; Litt, 2007). Predicted euFUL protein sequences are more similar in sequence to predicted FUL-like proteins sequences (and hence their name) found in non-core eudicots, whereas there is evidence supporting the sequence divergence of the euAP1 proteins (Litt and Irish, 2003; Vandenbussche et al., 2003; Litt, 2007; Shan et al., 2007). All euFUL and FUL-like proteins share a short conserved motif of six hydrophobic amino acids in which a tryptophan (in the fourth position) seems to be strictly conserved (Litt and Irish, 2003; Vandenbussche et al., 2003; Litt, 2007; Shan et al., 2007). The function of this motif is unknown. The euAP1 proteins are characterized instead by an acidic transcription activation motif, which has been shown to activate transcription in a yeast system (Riechmann et al., 1996; Cho et al., 1999) and a farnesylation motif (CaaX) (a signal for a post-translational modification of the protein by the attachment of an isoprenoid molecule to the C-terminal cysteine residue) hypothesized to be important in mediating multi-protein complex formation (Yalovsky et al., 2000). The presence of these divergent functional motifs is consistent with the idea that sequence modification that occurred after the core-eudicot duplication conferred new functional capabilities in the euAP1 proteins, different from those attributed to the euFUL and FUL-like proteins.

Additional duplication events occurred in specific lineages of core and non-core eudicots. One such duplication occurred early in the evolution of the *euFUL* clade at the base of the core eudicots producing two clades: *euFUL1* and *euFUL2* (Litt and Irish, 2003; Shan et al., 2007; Fig. 1.2). As a result, in general, most eudicots would be expected to have three *AP1*/*FUL* paralogs, one belonging to the *euFUL1* clade, another one belonging to the *euFUL2* clade, and another one belonging to the *euAP1* clade (Fig. 1.2). However *AP1/FUL* genes in other core eudicots have undergone independent local (taxon-specific) duplications and loss resulting in different numbers of copies. *Arabidopsis* possesses four *AP1/FUL* genes: *AP1*, another *euAP1* paralog known as *CAL* (*CAULIFLOWER*)(Kempin et al., 1995; Lowman and Purugganan, 1999), *FUL,* and another *euFUL* named *AGL79*. *AGL79* has diverged extensively in terms of sequence, expression patterns and presumably function (Parenicová et al., 2003). As a result, *Arabidopsis* has a unique combination of gene copies compared to the rest of the core eudicots. Similar scenarios occur in other Rosids, like Fabaceae (Berbel et al., 2001; Taylor et al., 2002; Benlloch et al., 2006) and in Asterids, for instance in Solanaceae (Hileman et al., 2006; Leseberg et al., 2008), where some species like tomato have 1 *euAP1* and 5 *euFUL* gene copies, or petunia lacking a *euAP1* member and with 3 *euFUL* gene copies. This creates difficulties for extrapolating and even comparing the function of *AP1*/*FUL* genes in core eudicots with that of their orthologs in non-core eudicots.

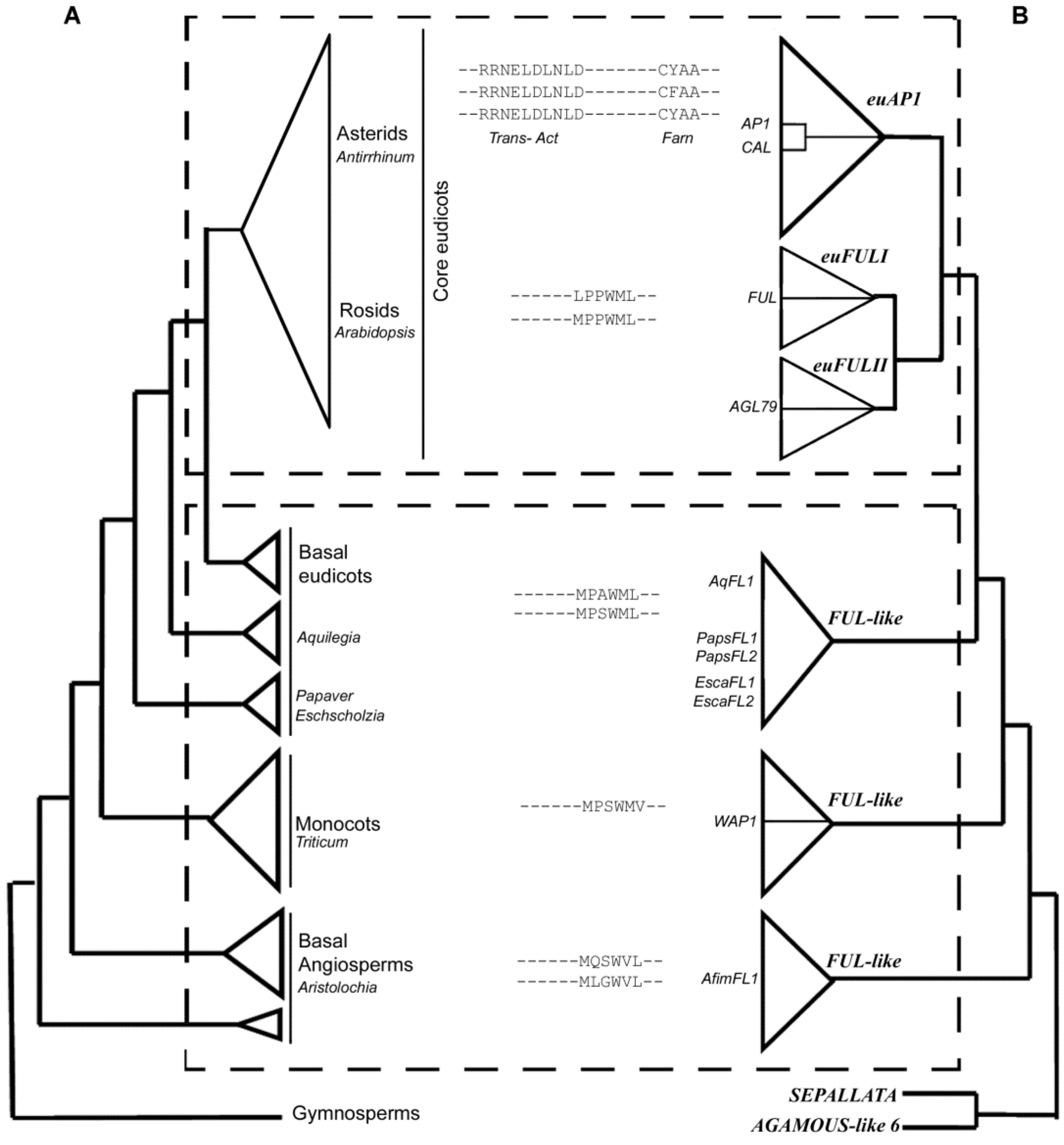

Figure 1.2. Simplified angiosperm phylogeny (A) paired with the gene phylogeny of *AP1*/*FUL* homologs (B) and their C-terminal motifs (based on Litt and Irish, 2003).

The angiosperm phylogeny shows the five major lineages (i.e. basal angiosperms, monocots, basal eudicots, rosids and asterids) and indicates the phylogenetic position of *Antirrhinum* (snapdragon), Arabidopsis, *Aristolochia* (Aristolochiaceae), *Eschscholzia* and *Papaver*

(Papaveraceae) and *Triticum* (wheat; Poaceae) The simplified *AP1/FUL* gene lineage tree (B) shows the core-eudicot gene duplication. The lower dotted box indicates the groups in which *FUL-like* genes are found, which includes all taxa outside of the core eudicots. The upper dotted box shows the *euAP1* and *euFUL* genes in core eudicots. Arabidopsis *AP1/FUL* homologs (*AP1*, *CAL*, *FUL*, *AGL79*) are shown in the gene tree, as are *PapsFL1*, *PapsFL2*, *EscaFL1*, and *EscaFL2*. In the center are the C-terminal protein motifs typical of the gene groups depicted in (B): the euAP1 transcriptional activation (Trans-Act) and farnesylation (Farn) motifs and the FUL-like motif characteristic of euFUL and FUL-like proteins.

FUL-like genes in non-core eudicots have also gone through lineage-specific duplication events. For instance, three clades of *FUL-like* genes are recognized in grasses (Preston and Kellogg, 2006, 2007) and different numbers of copies have been isolated from other monocots (Litt and Irish, 2003; Tsaftaris et al., 2004; Chen et al., 2008) and basal eudicots (Litt and Irish, 2003; Shan et al., 2007; Liu et al., 2010). As expected all sequences have the conserved FUL-like motif and lack the euAP1 functional motifs (reviewed in (Litt, 2007), which suggests that *FUL*-like genes might have different functional capabilities compared with those of *euAP1*genes.

1.3. Expression of *AP1/ FUL* genes

Gene expression analyses help to elucidate the spatial and temporal domains of activity of particular genes. Expression patterns of transcription factors have been frequently used to generate hypotheses about their functions. In this section, an overview of what is known about expression of *AP1/FUL* genes in plants is presented (Fig. 1.3).

In *Arabidopsis APETALA1* and *FRUITFULL* expression is mutually exclusive. While *AP1* is restricted to the floral meristem and is later localized to emerging sepals and petals (Irish and Sussex, 1990; Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1993; Yanofsky, 1995;

Ferrandiz et al., 2000; Blázquez et al., 2006), *FUL* is expressed in the inflorescence meristem, cauline leaves, carpels and fruit, particularly in the valve tissue (Gu et al., 1998; Ferrandiz et al., 2000; Blázquez et al., 2006). Expression of other *euAP1* genes in core eudicots such as *SQUA* in *Antirrhinum* (Huijser et al., 1992) *PEAM4* in *Pisum* (Berbel et al., 2001; Taylor et al., 2002) and *LeMADS_MC* in *Solanum* (Vrebalov et al., 2002) is also restricted to the floral meristem and the two outer floral whorls, although *SQUA* expression has also been detected in carpels and bracts (Huijser et al., 1992) and *LeMADS_MC* in carpels (Vrebalov et al., 2002). *AP1* orthologs in other core eudicots such as *Silene* (Caryophyllaceae) and *Nicotiana* (Solanaceae) are expressed more broadly, in most floral organs (Hardenack et al., 1994; Wu et al., 2000) and in *Silene* expression of *SLM4* (the *euAP1* ortholog) is also found in the inflorescence meristem (Hardenack et al., 1994). The most divergent expression profile documented to date for this gene clade is that of *GSQUA* from *Gerbera* (Asteraceae), where the *euAP1* ortholog is found throughout developing vascular traces in the flower (Yu et al., 1999); Fig. 1.3).

Similar to *FUL*, other core-eudicot *euFULI* and *euFULII* genes are expressed in the inflorescence apical meristem and often strongly in carpel walls; however, expression is often broader, frequently in vegetative meristems, leaves, stems and bracts, as well as sepals and petals (Immink et al., 1999; Yu et al., 1999; Wu et al., 2000; Elo et al., 2001; Muller et al., 2001; Shchennikova et al., 2004; Yu et al., 2008; Sather and Golenberg, 2009) (Fig. 1.3).

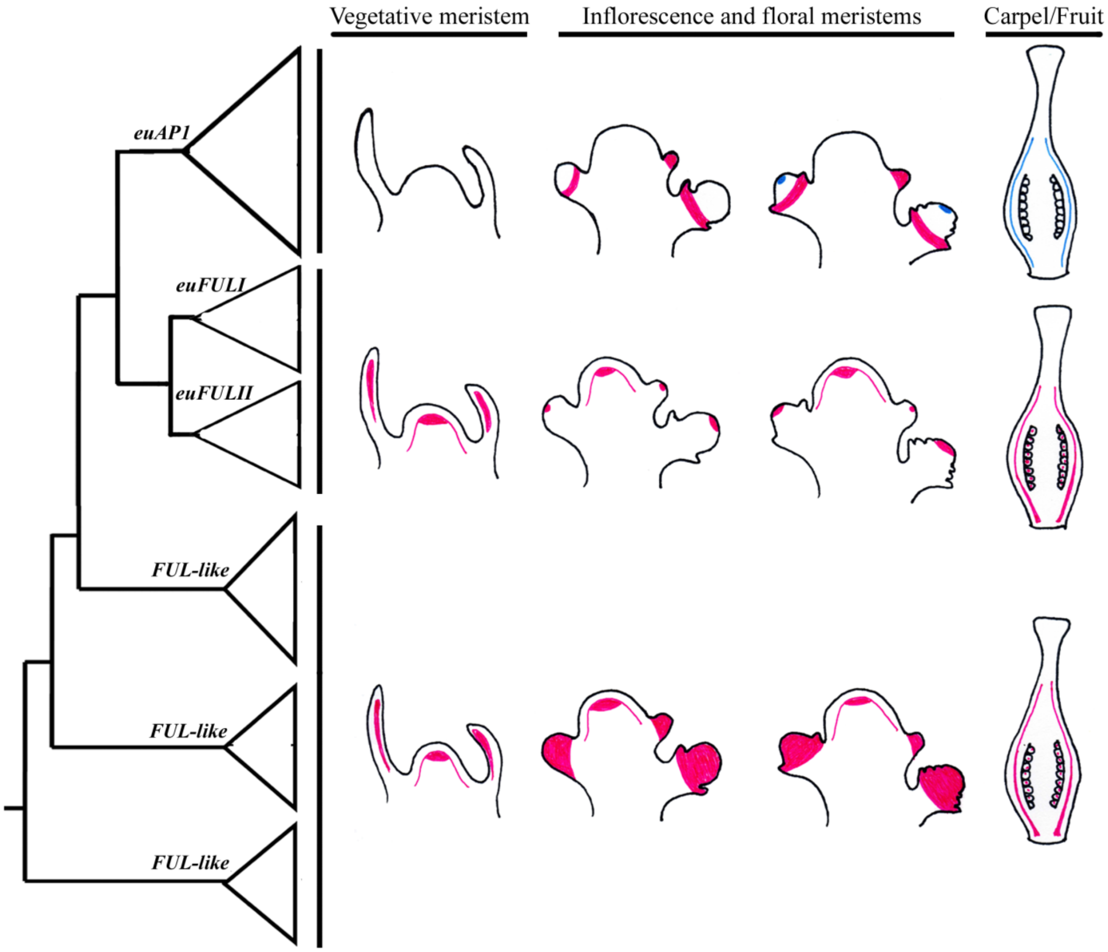

Figure 1.3. Expression patterns of *AP1/FUL* genes.

Typical expression patterns reported for *euAP1*, *euFUL* and *FUL-like* genes are in pink. More uncommon expression patterns reported for *euAP1* genes are in blue. Note that expression patterns of the pre-duplication *FUL-like* genes include all those reported for *euFUL* and *euAP1* genes after the duplication.

FUL-like genes in non-core eudicots also have broader expression patterns than eu*AP1*. *FUL-like* genes can be expressed in vegetative as well as in reproductive meristems, in leaves, modified protective leaves (cataphylls) and bracts, in early floral primordia, all floral parts, modified floral parts (lemma, palea and lodicules in grasses, and gynostemium in orchids), and fruits (Fig.1.3)

(Yu and Goh, 2000; Gocal et al., 2001; Pelucchi et al., 2002; Tsaftaris et al., 2004; Kim et al., 2005; Adam et al., 2007; Preston and Kellogg, 2007; Wu et al., 2007; Chen et al., 2008).

Several issues can be highlighted from the expression data. Few *euAP1* orthologs within the core eudicots have the same expression profiles as *AP1* in *Arabidopsis*. Besides the perianth, they are often expressed in carpels, and expression may be found in stamens, bracts and inflorescence meristems. Roles for *euAP1* genes in floral meristem identity and in perianth identity are consistent with the expression profiles; however, a role in carpel and fruit development is also plausible. In general, *euFUL* and *FUL-like* genes are found in vegetative as well as in reproductive tissues, in contrast with most *euAP1* genes. Expression in vegetative parts suggests *euFUL* and *FUL-like* genes could be involved in the transition from vegetative to reproductive meristem. Since the *euAP1* genes are expressed more restricted than the *euFUL* and *FUL-like* genes, it can be hypothesized that sequence divergence in the *euAP1* clade after the core-eudicot duplication event resulted in a change in regulation of *euAP1* orthologs. Thus resulting in different capabilities and possibly in different developmental mechanisms in core eudicots vs. non-core eudicots. As will be explained in the following sections, functional data are critical to better understanding of the data from the expression patterns and to strengthen the hypothesis that gene duplication events do impact functional evolution in the *AP1/FUL* gene lineage.

1.4. Function of *AP1/FUL* genes

Most of what is known about the function of *euAP1* and *euFUL* genes comes from *Arabidopsis*. Specification of proper floral meristem identity and proper identity of the first and second floral whorls (sepals and petals respectively) is attributed to *AP1* since both floral meristem identity and perianth identity are disrupted in *ap1* mutants of *Arabidopsis* (Irish and Sussex, 1990; Bowman et al., 1991; Coen and Meyerowitz, 1991; Yanofsky, 1995; Ferrandiz et al., 2000) (Figure 1.4). In *ap1* plants sepals are replaced by bract-like structures, petals do not develop, and the second whorl is occupied by secondary flowers subtended by the organs of the first whorl (Irish and Sussex, 1990; Bowman et al., 1991; Coen and Meyerowitz, 1991; Yanofsky, 1995; Ferrandiz et al., 2000). Consistent with the loss of floral identity, tertiary flowers can initiate in the axils of the first whorl organs of the secondary flowers giving rise to a dramatic reiterative branching pattern. Despite the remarkable modification in the floral groundplan, the two inner whorls retain their identity forming normal stamens and carpels. *Arabidopsis cal* (the *euAP1* paralog) mutants do not have a distinct phenotype different from wild type plants; however, in the *ap1 cal* double mutants the branching pattern is intensified and floral meristems are replaced by inflorescence meristems that provide the plant with a "cauliflower" appearance (Irish and Sussex, 1990; Coen and Meyerowitz, 1991; Bowman et al., 1993; Kempin et al., 1995; Yanofsky, 1995; Ferrandiz et al., 2000). Similarly, *Arabidopsis ap1 cal ful* triple mutants do not flower but instead branch repeateadly to form leafy shoots (Ferrandiz et al., 2000). *CAL* is also required for proper petal identity under certain conditions, as shown by experiments in which constitutive expression of *SEP3* rescues petals in the *ap1* mutant but not in the *ap1cal* mutant (Castillejo et al., 2005).

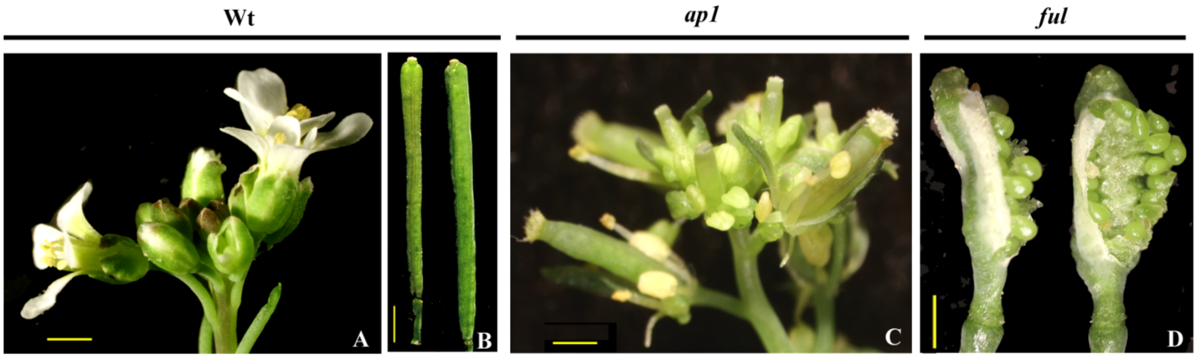

Figure 1.4. *Arabidopsis ap1* and *ful* mutants.

A. Wild type *Arabidopsis* flowers. **B**. Wild type *Arabidopsis* fruits. **C**. The *ap1 Arabidopsis* mutant showing homeotic conversion of sepals into bracts with axillary meristems and the loss of petals. **D**. The *ful Arabidopsis* mutant showing shorter, fatter, prematurely dehiscent fruits.

It has been postulated that *AP1* exerts its effect on floral meristem identity through repression of *AGAMOUS-like 24* (*AGL24*) (Yu et al., 2004; Gregis et al., 2006) which promotes inflorescence identity. *AGL24* is normally expressed in the vegetative shoot apex and up-regulated in the inflorescence apex during the floral transition. In *ap1* mutants, *AGL24* is expressed throughout the floral meristem and is thought to confer the inflorescence-like structure to the outer whorls (bracts subtending secondary flowers).

Other *euAP1* mutants show a different phenotype from *ap1.* The *squa* mutants in snapdragon (Huijser et al., 1992), *pim* mutants in pea (Berbel et al., 2001; Taylor et al., 2002) and *lemads-mc* mutants in tomato (Vrebalov et al., 2002), lack proper specification of floral meristem identity and produce a repetitive branching pattern in the inflorescence associated with the formation of leafy shoots and bracts similar to *ap1*. However, occasionally floral primordia are formed from which complete flowers with mainly normal organ identity are formed (Huijser et al., 1992; Berbel et al., 2001; Taylor et al., 2002; Vrebalov et al., 2002), thus sepals and petals appear to be specified normally when they are formed. None of these phenotypes evidence the putative role of *euAP1* genes in specifying the identity of the two whorls of perianth (A-function), but rather support the idea that *euAP1* genes are required for the proper transition from a branched inflorescence to a flower with concurrent sepal formation.

Other evidence of the role of *AP1* orthologs comes from heterologous transformation experiemnts. Over-expression of *euAP1* genes from pea and chrysanthemum in *Arabidopsis* results in early flowering and occasionally in leaf defects (Berbel et al., 2001; Elo et al., 2001; Jang et al., 2002; Taylor et al., 2002; Shchennikova et al., 2004; Flachowsky et al., 2007). However, early flowering is a very common phenotype that results from over-expression of MADS-box genes. When ectopically expressed in *Arabidopsis, NtMADS11,* a *euAP1* gene from tobacco is able to partially complement the *ap1-1* mutant and completely or partially restore petal identity (Jang et al., 2002; Fornara et al., 2004). Ectopic expression of *BpMADS3, 4* and *5* (the *euAP1* and two *euFUL Betula* [Betulaceae] orthologs respectively) in *Arabidopsis* also results in early flowering and the formation of a terminal floral structure (Elo et al., 2001; Flachowsky et al., 2007). Ectopic expression of *BpMADS3, 4* and *5* in tobacco plants results in early flowering and leaf defects (droopy leaves), but flower and inflorescence structure remain unaffected (Elo et al., 2001; Flachowsky et al., 2007). In general, these results suggest that there might be some functional conservation between *euAP1, euFUL* and *FUL-like* genes in spite of the sequence divergence. However, it is important to highlight that when function is assessed in a different genetic background, prediction of endogenous roles can be misleading. In general, a role for *euAP1* genes in meristem identity seems to be conserved; but their role in sepal and petal identity is not consistently supported by the data.

FUL is redundant with *AP1* and *CAL* in proper floral meristem identity, as shown by the triple mutants *ap1 cal ful Arabidopsis* (Ferrandiz et al., 2000). In addition, *FUL* also exerts a unique function in fruit development (Figure 1.4). The *ful* mutants in *Arabidopsis* lack coordinated growth of the fruit tissue leading to crowded seeds, failure in dehiscence, and premature rupture of the carpel valves (Gu et al., 1998; Ferrandiz et al., 2000). *FUL* is required for normal cellular differentiation in the epidermal cells of the valves a very different function from *AP1* (Gu et al., 1998; Ferrandiz et al., 2000). Over-expression of the *Antirrhinum euFUL* paralog *DEFH28* in *Arabidopsis* causes loss of the identity of valve margins and replum, resulting in the absence of a dehiscence zone and failure to dehisce (Muller et al., 2001), suggesting a role in proper fruit formation. Furthermore, showed a reduction in number of rosette leaves accompanied by early flowering and an abnormal floral structure terminating the shoot apex, indicating a role in early flower development similar to *FUL* (Muller et al., 2001). In petunia (Solanaceae), silencing of one *euFUL* paralog, *PFG,* results in plants that remained vegetative (Immink et al., 1999). In general, functional evidence suggests that *euFUL* genes in other core eudicots have the same dual roles as *FUL*: an early role in promoting the transition to reproductive meristems and a late role in proper fruit development.

Functional studies of *AP1/FUL* genes outside the core eudicots are scarce. A *ful-like* mutant is only known from wheat. Mutants of *WAP1*, one of three *FUL-like* paralogs, remain in the vegetative stage after vernalization. This suggests a role in the proper transition to flowering, earlier in the reproductive transition than *AP1* and *FUL* (Murai et al., 2003; Trevaskis et al., 2003; Yan et al., 2003; Trevaskis et al., 2007). Studies on the molecular basis of vernalization-induced flowering in cereals (Trevaskis et al., 2003; Trevaskis et al., 2007; Preston and Kellogg, 2008) suggest that *FUL-like* genes in these species are up-regulated in leaves and meristems before the emergence of the spikelet primordium and therefore might function as an early signal of the transition to flowering. Assays via heterologous over-expression in *Arabidopsis* suggest that *OsMADS14* from *Oryza* (Fornara et al., 2004), *LtMADS15* from *Lolium* (Gocal et al., 2001), and *LMADS6* and *LMADS7* from *Lilium* (Chen et al., 2008) driven by the CaMV35S promoter are able to partially complement strong *ap1* mutants, suggesting that, in spite of the differences in C terminal domains, functional capabilities between *FUL-like* genes and *euAP1* genes might be conserved to some extent. Ectopic expression of *LMADS5, LMADS6,* and *LMADS7* from *Lilium* in wild type *Arabidopsis* resulted in plants with early flowering after the formation of only six curled rosette leaves and the formation of terminal flowers (Chen et al., 2008). Additionally homeotic conversion of sepals into carpels and petals into stamens was observed, suggesting misregulation of C class genes in the outer two whorls (Chen et al., 2008).

In the ABC model, the A-function (specification of the proper identity of sepals and petals) was attributed to *AP1*, since perianth identity was disrupted in the *ap1* mutant of *Arabidopsis* (Irish and Sussex, 1990; Coen and Meyerowitz, 1991; Mandel et al., 1992; Bowman et al., 1993; Yanofsky, 1995). The data available suggest however, that although widely recognized, this A-function is not conserved in other taxa outside the Brassicaceae. Mutants of eu*AP1* orthologs from other core eudicots do not lose the identity of both whorls of perianth organs, despite the loss of meristem identity (Huijser et al., 1992; Berbel et al., 2001; Taylor et al., 2002; Vrebalov et al., 2002; Benlloch et al., 2006). *Arabidopsis* possesses a unique gene complement, with four paralogs (Kempin et al., 1995; Lowman and Purugganan, 1999; Parenicová et al., 2003; Alvarez-

Buylla et al., 2006; Blázquez et al., 2006) and is possible that differential subdivision of function in these genes confers different functional capabilities to *AP1/FUL* paralogs in this species.

Some functions are overlapping between *euFUL* and *FUL-like* genes such as the proper transition reproduction in Petunia and wheat (Immink et al., 1999; Danyluk et al., 2003; Murai et al., 2003). However, the extent to which this function is conserved in other *euFUL* and *FUL-like* genes is not known due to the lack of functional data for *FUL-like* genes from magnoliids and basal eudicots and other *euFUL* genes in core eudicots. It is clear that an understanding of the functional evolution of the *AP1/FUL* genes, and the effect of the core-eudicot duplication, requires further examination of the endogenous roles of *FUL-like* genes from a wider spectrum of phylogenetically informative species.

1.5. Protein- protein interaction data from AP1/FUL proteins

It has been hypothesized that different combinations of MADS-domain-containing proteins activate different groups of target genes in each floral whorl (Theissen and Saedler, 2001; Melzer and Theissen, 2009; Melzer et al., 2009). In yeast, MADS-domain-containing proteins have been shown to form ternary and quaternary complexes (Riechmann et al., 1996; Egea-Cortines et al., 1999; Pelaz et al., 2001; Immink et al., 2003; DeFolter et al., 2005; Leseberg et al., 2008) potentially providing specificity for the recognition and transcription activation of target genes (Immink et al., 2002; Melzer and Theissen, 2009; Melzer et al., 2009; Immink et al., 2010). Since protein-protein interaction has been hypothesized as critical for *in vivo* roles of the MADS box proteins, changes in protein interaction imply possible changes in function during development. Protein partners for AP1/FUL proteins in *Arabidopsis* have been identified in yeast (Riechmann et al., 1996; Pelaz et al., 2001; DeFolter et al., 2005); Fig. 1.5), but partners of other euAP1 or euFUL proteins have been much less studied (but see Immink et al., 2003; Leseberg et al., 2008) (Figure 1.5). Information from FUL-like proteins is scarce and mostly consists of heterologous interactions with *Arabidopsis* proteins (Moon et al., 1999; Ciannamea et al., 2006); Fig. 1.5). Study of the interacting partners of FUL-like proteins will provide additional information on conservation and evolution of function. If these proteins interact with different partners from the ones known in *Arabidopsis that* would suggest changes in the role of this group of proteins in non-core eudicot flower development.

In *Arabidopsis AP1* is regulated indirectly by different interacting pathways involved in the transition to flowering; these are the photoperiod pathway, the vernalization-response pathway, the autonomous pathway and the gibberellin signaling pathway (Koornneef et al., 1998; Mouradov et al., 2002; Ratcliffe and Riechmann, 2002; Simpson and Dean, 2002; Putterrrill et al., 2004). These pathways converge on a few key MADS-box transcription factors such as *FLC* (*FLOWERING LOCUS C*), *FT* (*FLOWERING LOCUS T*) and *SOC1* (*SUPRESSOR OF CONSTANS1*) which are known to activate floral meristem genes and floral identity genes such as *AP1* and *LFY* (*LEAFY*; see revision in (VanStraalen and Roelofs, 2006). In yeast, protein-protein interaction has been shown to occur between both AP1 and SOC1 as well as between FUL and SOC1 (DeFolter et al., 2005) (Fig. 1.5). AP1 and FUL also interact with proteins involved in floral meristem and later floral organ identity such as AGL6, AGL24 and SVP (*Arabidopsis* paralogs from a recent duplication), AGL21, and SEP1/3/4 (DeFolter et al., 2005; Gregis et al., 2006).

The Arabidopsis euAP1 paralog CAL interacts with a subset of the AP1 interactors involved in meristem identity (Pelaz et al., 2001). Among the exclusive partners of AP1 are several MADS box proteins involved in floral organ identity such as AP3 and PI (Riechmann et al., 1996; DeFolter et al., 2005). In yeast studies AG was shown only to interact with FUL (DeFolter et al., 2005); AP1 was shown to interact with AG by in vitro immunoprecipitation (Riechmann et al., 1996) but the dimer could not bind DNA. Additionally, it has been shown, by in vitro immunoprecipitation and in yeast, that AP1 and the *Antirrhinum* ortholog SQUA homodimerize and bind DNA (Riechmann et al., 1996; Honma and Goto, 2001; DeFolter et al., 2005). FUL has not been shown to homodimerize.

euAP1 and euFUL protein interactions have also been assayed in *Petunia*, *Antirrhinum*, *Nicotiana*, *Solanum* and *Chrysanthemum* using the yeast system (Fig. 1.5). In general these results are partially consistent with those from *Arabidopsis*. AP1 and FUL orthologs have been shown to interact with orthologs of SEP1, SEP3, SOC1, and AGL24/SVP in these species, and less frequently to interact with AP3/PI or AG genes, suggesting similar functional roles to AP1 and FUL (Egea-Cortines et al., 1999; Immink et al., 2002; Immink et al., 2003; Shchennikova et al., 2004; DeFolter et al., 2005; Leseberg et al., 2008). However, euFUL and euAP1 paralogs often have similar but not identical interactions, as has been illustrated in tomato. Tomato has five *AP1/FUL* genes, of which the euFUL proteins SlMBP20 and SlMBP7 have a larger number of interactions than TM4. In addition whereas TM4 (euFUL) can bridge interactions between SEP and AGL24 genes (TM4- RIN/MADS1 - SlMBP24), LeMADS_MC (euAP1) has been shown to bridge interactions between SEP and AG genes (MC- TM5- TAG1/TAGL11;(Leseberg et al., 2008).

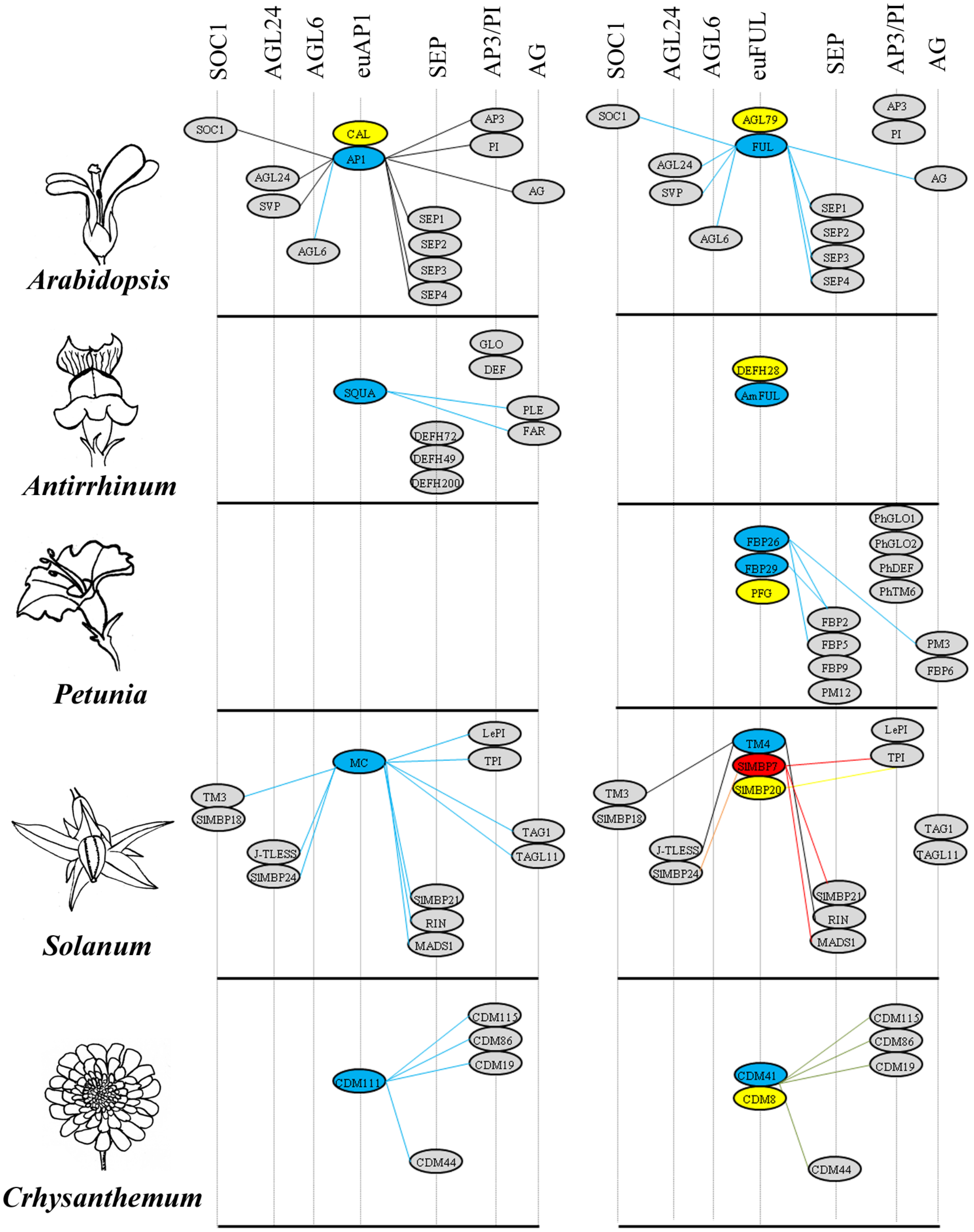

Figure 1.5. Interactions of euAP1 and euFUL proteins from several species.

In general euAP1, and euFUL proteins commonly interact with SEP, AGL6, AGL24 and SOC1 proteins, and less frequently with AP3/PI and AG proteins. No euAP1 orthologs have been identified in Petunia yet, all copies in this species possess the FUL motif and cluster with FUL in phylogenetic analyses (Immink et al., 2003; Rijpkema et al., 2006). Two Antirrhinum euFUL proteins have been identified (Muller et al., 2001; Litt and Irish, 2003), but no interactions have been tested between these and other MADS-box proteins. Lines among proteins indicate a positive interaction. Absence of lines indicates that interactions were tested and were negative. AP1/FUL proteins are colored in blue, yellow or red to distinguish among paralogs. When an interaction has been shown to occur for all paralogs is black, when it has been shown to occur only for two out of three copies is in orange (for yellow and red proteins) or green (for yellow and blue proteins), when the interaction is copy-specific is labeled with its same color (blue, yellow or red).

FUL-like protein interaction data have been reported for *Lolium perenne* and *Oryza sativa.* In *Lolium,* LpMADS1, LpMADS2 and LpMADS3 FUL-like proteins were tested in a yeast two hybrid screen. LpMADS1 and LpMADS2 are able to form homodimers and heterodimers with each other while LpMADS3 does not seem to form dimers or interact with its homologs (Ciannamea et al., 2006). Also a heterologous yeast two hybrid screening was performed with the *Lolium* FUL-like proteins and a set of *Arabidopsis* proteins. Interestingly, LpMADS1 and LpMADS2 can interact with FUL, AGL24, several C-class proteins (AG, SHP1, SHP2), E-class proteins (SEP1, SEP2, SEP4), and proteins involved in floral transition (SOC1, SVP; (Ciannamea et al., 2006). *Oryza* FUL-like proteins were found to interact with orthologs of AGL6, SEP1, and AGL24/SVP (Moon et al., 1999). In neither species were interactions with AP3 or PI orthologs reported. This suggests that FUL-like proteins could interact in a fashion more similar to FUL than to AP1 and therefore those functional capabilities are conserved between FUL-like and euFUL proteins, but diverged in euAP1 proteins.

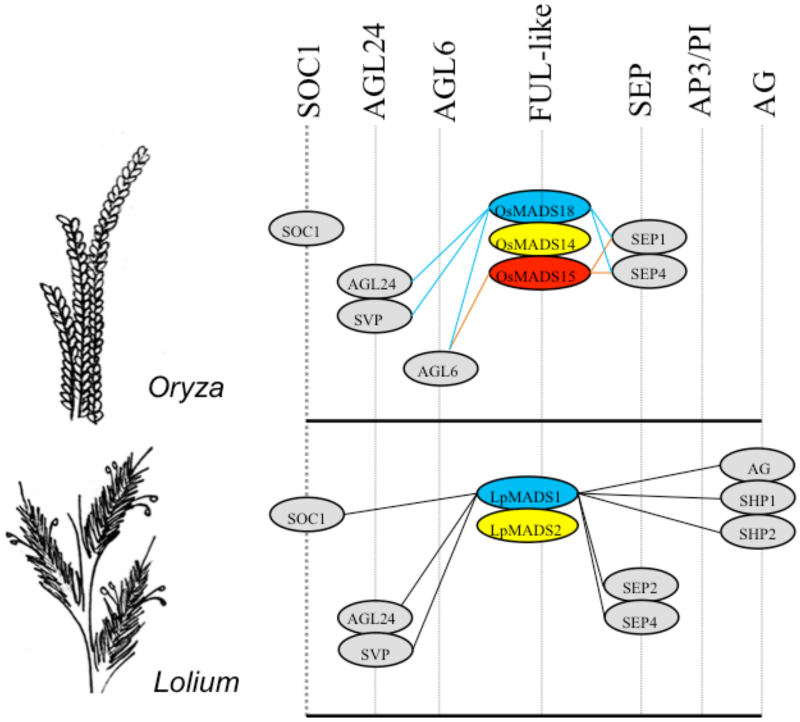

Figure 1.6. Interactions of FUL-like proteins from grasses based on (Moon et al., 1999; Ciannamea et al., 2006).

Lines among proteins indicate a positive interaction, Absence of lines indicates interactions were tested and were negative. AP1/FUL proteins are colored in blue, yellow or red to distinguish among paralogs. When an interaction has been shown to occur for all paralogs is black, when it has been shown to occur only for two out of three copies is in orange (for yellow and red proteins) or green (for yellow and blue proteins), when the interaction is copy-specific is labeled with its same color (blue or red).

This dissertation implemented the yeast two hybrid system to evaluate protein-protein interaction between FUL-like proteins and other MADS-box proteins involved in floral transition, such as AGL6, AGL24, SOC1, SEP1/3/4, as well as MADS box proteins involved in floral organ identity, such as AP3, PI and AG in *Aquilegia coerulea*. Since protein dimers or quartets are thought to be the basis of MADS protein function, the establishment of interacting protein partners for FUL-like proteins in the light of what is known for FUL in *Arabidopsis* will provide additional evidence about their function divergence or conservation. Although the yeast two hybrid system does not provide evidence of the protein interactions in the plant, it can at least address whether potential interaction can occur between protein pairs, trimers or tetramers. Despite the artifacts that the system produces, most of the available data for MADS-box proteins interactions come from yeast experiments, providing a framework for comparison with published data from other plant groups.

1.6. Research questions: the functional evolution of the *AP1/FUL* gene lineage

This manuscript addresses three main questions regarding the evolution of *AP1/FUL* genes:

1. What is the expression of *FUL-like* genes in basal angiosperms and basal eudicots?. Based on the expression patterns of these genes, can their function be hypothesized?

It is known that *FUL-like* genes are broadly expressed compared with *euAP1* genes (Fig. 1.3). On the basis of the expression patterns, particularly in vegetative organs, an early role in the vegetative to reproductive transition has been hypothesized for *FUL-like* genes, in contrast to the later role in floral meristem identity attributed to *euAP1* genes. Expression does not rule out roles in perianth identity, and in carpel and fruit development, functions that have been identified in *AP1/FUL* core-eudicot homologs. Previous studies have in general not looked in detail at temporal and spatial expression, and have not looked at expression in all paralogs of a particular species (but see Preston and Kellogg, 2007). Analyses of expression patterns using RT-PCR and *in situ* hybridization are used here to record expression patterns of these pre-duplication genes, are compared with those of other species, and are linked with functional studies to evaluate potential conservation of expression and function. Results of *FUL-like* genes expression patterns are shown for *Papaver somniferum* and *Eschscholzia californica* in chapter 3 and for *Aquilegia coerulea* in chapter 4.

2. What is the function of *FUL-like* genes?; How does it compare with the roles of *euFUL* and *euAP1* genes?. What are the implications on the functional evolution of the gene lineage?

The *euAP1* genes appear to be important for floral meristem identity and possible perianth identity. The *euFUL* genes are thought to be important for floral meristem identity and proper fruit development in *Arabidopsis* (Irish and Sussex, 1990; Gu et al., 1998; Ferrandiz et al., 2000) and *Antirrhinum* (Huijser et al., 1992; Muller et al., 2001) (Fig. 1.4). Very little is known about *FUL-like* genes, but they may play a role in the floral transition in wheat (Danyluk et al., 2003; Murai et al., 2003; Trevaskis et al., 2003; Trevaskis et al., 2007). Based on expression profiles, other putative roles have been attributed to *FUL-like* genes, particularly from grasses: inflorescence and floral meristem determinacy (Preston and Kellogg, 2007, 2008), floral transition (Gocal et al., 2001; Pelucchi et al., 2002; Danilevskaya et al., 2008; Preston and Kellogg, 2008) and carpel or fruit development (Preston and Kellogg, 2007). Down-regulation of the endogenous levels of *FUL-like* genes in *P. somniferum*, *E. californica* and *A. coerulea* was used to test the hypothesis that these genes play a role in the floral transition or in floral development. Phenotypes resulting from the down-regulation of *FUL-like* genes in *P. somniferum* and *E. californica* are presented in chapter 3 and for *A. coerulea* in chapter 4. These results shed light on the functional consequences of the core-eudicot gene duplication and the universality of the ‘A-function’ in other flowering plants outside the Brassicaceae.

3. Which proteins interact with FUL-like proteins?. How do these interactions compare with the post-duplication euFUL and euAP1 protein interactions?

Changes in the interaction partners of different AP1/FUL orthologs suggest divergence in their biochemical function. Yeast two hybrid protein-protein interaction screenings in *Arabidopsis* suggest that FUL and AP1 are part of both the flower induction network and the flower organ formation network. Although each protein has some different interaction partners, both overlap in the interaction with SEP1/3/4, AGL6, AGL24 and AG, proteins that are associated with transition to flowering, flower and inflorescence meristem identity, and floral organ identity (Bowman et al., 1991; Pelaz et al., 2000; Zahn L.M., 2005; Gregis et al., 2006; Zahn, 2006; Schauer et al., 2009). This dissertation presents data on the interaction of FUL-like proteins with other MADS-box proteins in *Aquilegia coerulea*. Yeast two hybrid analyses are used to identify interactions of the FUL-like protein from *Aquilegia* with orthologs of proteins shown to form complexes with AP1 or FUL in *Arabidopsis.* The results are shown in chapter 4 and are important to determine if the pre-duplication FUL-like proteins have the same interaction capabilities as the core-eudicot euAP1 and euFUL proteins (Fig. 1.5).

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CHAPTER TWO

TAXA SELECTED

With the purpose of investigating the function of *FUL-like* genes in non-core eudicots, three species (Table 1) belonging to two families of the basal eudicot grade were chosen: *Eschscholzia californica* Cham. and *Papaver somniferum* L. (Papaveraceae) and *Aquilegia coerulea* E. James (Ranunculaceae). These species are easy to germinate and grow, are herbaceous and have short life cycles. In addition, the *Aquilegia* genome has been sequenced (http://www.phytozome.net) all have a large amount of EST data and most of them also have proven to be amenable to emerging PTGS (Post Transcriptional Gene Silencing) techniques (Becker et al., 2005; Hileman et al., 2005; Drea et al., 2007; Gould and Kramer, 2007; Kramer et al., 2007; Wege et al., 2007; Orashakova et al., 2009).

Basal eudicots exhibit a highly variable floral groundplan (Endress, 1994, 2001; Ronse De Craene et al., 2003; Soltis et al., 2005; Ronse De Craene, 2010). Flowers of *Papaver* and *Eschscholzia* are hypogynous and possess a dimerous perianth with free parts, a high number of spirally arranged stamens and a syncarpous gynoecium which becomes a capsule (Ronse De Craene and Smets, 1990; Becker et al., 2005; Kadereit and Erbar, 2011). *Aquilegia* has hypogynous flowers with a tepaloid, pentamerous perianth with free parts, highly modified petals and a high number of spirally arranged stamens and an apocarpous gynoecium that forms follicular fruits (Tepfer, 1953; Hodges, 1997; Tucker and Hodges, 2005). All the characters mentioned above vary among Ranunculales (see chapter 5) and contrast strongly with the fixed whorled floral structure, with few floral parts and a predominantly syncarpous gynoecium, common in core eudicots (Endress, 1994, 2001; Soltis et al., 2003; Soltis et al., 2005; Ronse De Craene, 2010).

Table 2.1. Summary of the main morphological differences between selected taxa for the project.

Species	***Papaver somniferum***	***Eschscholzia californica***	***Aquilegia coerulea***
Inflorescence	One terminal (usually solitary) flower.	Cymes. Monopodial growth.	Cymes. Sympodial growth.
Perianth symmetry	Disymmetric.	Disymmetric.	Polysymmetric.
Floral Bud	Ovate and drooping or pendulous	Elongate and erect	Elongate and erect
Sepals	Two, free, not petaloid.	Two, fused, not petaloid.	Five, free, petaloid.
Petals	Four in two whorls. Outer narrower than inner petals.	Four in two whorls. Outer broader than inner petals.	Five, free, each with a nectar spur.
Stamens	Numerous, free, in several whorls.	Numerous, free, in several whorls.	Numerous, free, in 4-6 whorls. Inner staminodes persistent during fruit development.
Gynoecium	Six to eight fused carpels. Ovary superior with gynophore. Ovules numerous on parietal placentae.	Two fused carpels. Ovary superior with gynophore. Ovules numerous on parietal placentae.	Five free carpels. Ovary superior, apocarpous. Ovules numerous on submarginal placentae.
Fruit	Locular, poricidal capsule.. Seeds numerous.	Locular, septicidal capsule.. Seeds numerous.	Multiple follicles. Seeds numerous.

The selected taxa provide a broad framework for studying the expression and/ or function of *FUL*-like genes in plants that require vernalization (*Aquilegia*) versus plants that do not (*Eschscholzia* and *Papaver*), and plants with a syncarpous (*Eschscholzia* and *Papaver*) or apocarpous (*Aquilegia*) gynoecium resulting in different fruit types. All of the above are characteristics putatively related to functions of *AP1/FUL* genes as suggested by the direct role of the vernalization pathway in the regulation of *WAP1* (Moon et al., 1999; Danyluk et al., 2003; Murai et al., 2003; Trevaskis et al., 2003; Trevaskis et al., 2007), the multiple putative developmental roles of *euFUL* genes in inflorescence architecture (Ferrandiz et al., 2000; Berbel et al., 2001; Taylor et al., 2002; Benlloch et al., 2006), and the putative role of *FUL* in leaf and fruit formation in several core eudicots (Gu et al., 1998; Ferrandiz et al., 2000; Ferrandiz et al., 2000; Muller et al., 2001).

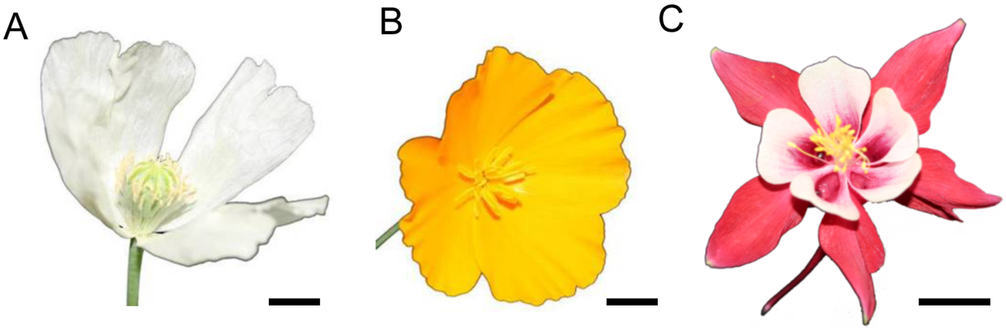

Figure 2.1. Taxa selected to assess the role of FUL-like genes. A *Papaver somniferum* (Papaveraceae). B. *Eschscholzia californica* (Papaveraceae). C *Aquilegia coerulea* (Ranunculaceae). Bars= 1cm.

This chapter will present a detailed description of the inflorescence, flower and fruit morphology and development of the four taxa selected. Emphasis will be given to species-specific features possibly linked with the role of *FUL-like* genes (branching, inflorescence structure, perianth and fruit development) that have not been described in published morphological or floral ontogeny studies.

2.1. ***Papaver somniferum*** **(Papaveraceae- Ranunculales)**

This species is an herb with simple alternate leaves. It is native to south-western Asia, central and southern Europe and Northern Africa (Carolan et al., 2006). Recent phylogenetic analyses confirm the traditional placement of *Papaver somniferum* within subsection *Papaver* s. str, in the subfamily Papaveroideae (Carolan et al., 2006). The species has been largely used as a system for investigating alkaloid production and in general secondary metabolites (Chitty et al., 2003; Carolan et al., 2006; Wijekoon and Facchini, 2011). It possesses a chromosome number of 2n= 22 (Hammer and Fritsch, 1977). Stable and transient transformation techniques have been standardized in this species (Chitty et al., 2003; Hileman et al., 2005). The latter have been implemented using *Papaver somniferum cv. persian white,* a small herb with a life cycle that is completed in 2-3 months. Seeds germinate in moist soil or vermiculite after a week. A week after germination the first true leaf expands. Leaves are entire to slightly lobed. Two types of leaves are produced: the larger rosette leaves produced in the vegetative stage, and the smaller alternate cauline leaves produced on the inflorescence axis. *P. somniferum* does not require vernalization to flower, and it does so eight weeks after germination. Only one terminal flower develops per plant. Several of the basal-most cauline leaves protect axillary buds that are usually dormant but can develop into axillary branches if for instance decapitation occurs. Floral buds are drooping or pendulous and only become erect just prior to anthesis.

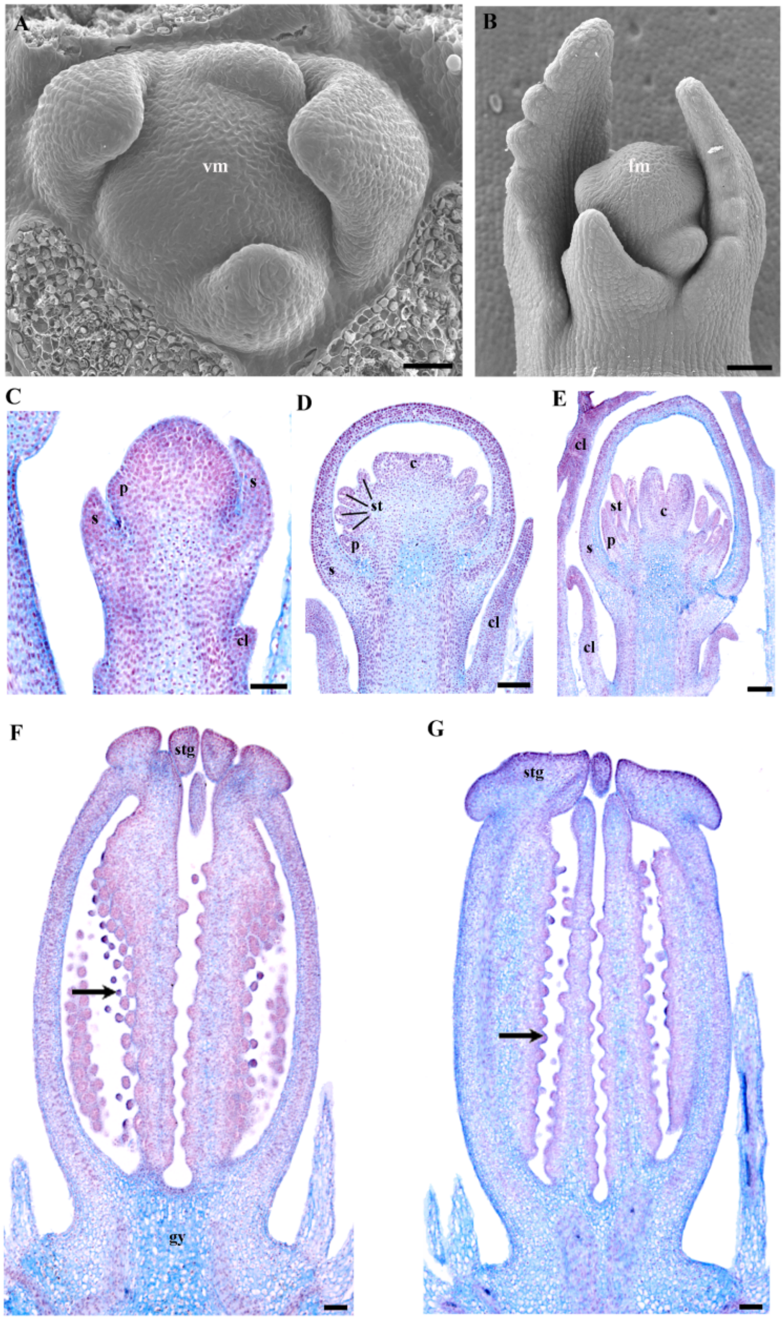

Figure 2.2. Flower development of *Papaver somniferum*.
(**A)** Vegetative meristem protected by the rosette leaves that arise spirally. **(B)** Floral meristem protected by the sub-opposite cauline leaves. **(C to E)** Flower development showing sepal (**s**) elongation and petal (**p**) primordia initiation **(C)**, followed by stamen (**st**) and carpel (**c**) primordia initiation **(D),** and elongation **(E). (F, G)** Oblong ovary on a short gynophore (**gy**) with a sessile peltate stigma (**stg**); the unilocular ovary is formed by six to eight carpels; ovules (arrows) have parietal placentation.

Flower development and late stages of fruit development, particularly those associated with the peak of alkaloids production and “lacerating” stages (i.e. when latex is extracted from the fruit), have been carefully characterized in this species (Ronse De Craene and Smets, 1990; Kapoor et al., 1995; Drea et al., 2007). The floral primordium is elliptic as the two sepal primordia initiate on the median plane. One of the sepals emerges first and tends to grow faster (Figure 2.2C). Later in development the flanks of both sepals become joined by inter-primordial growth (Ronse De Craene and Smets, 1990). When sepals start to cover the floral apex, two petal primordia initiate alternate with the sepals, and two other petals are almost simultaneously initiated opposite to the sepals. Petals are usually retarded in their growth. The first four stamen primordia initiate between the petals; soon more stamens proliferate acropetally in numerous whorls (Figure 2.2D). Development is completed when about 6 whorls have been initiated. Finally, a circular gynoecial primordium initiates (Figures 2.2D, E), followed by the formation of about 12 to 14 (fewer in the small persian white cultivar) septa. The ovary stands on a short gynophore, is globular to oblong, and possesses a sessile, peltate stigma that crowns the ovary (Figures 2.2F, G). The stigma is persistent through fruit development. A high number of ovules is produced in parietal placentation (Kadereit and Erbar, 2011).

Fruits are poricidal capsules that have been described as having an epidermis (exocarp) formed of thick densely packed parenchymatic cells, a mesocarp formed by collenchyma (toward the outside) and parenchyma (toward the inside) where vascular bundles and laticifers are abundant and a one-layer cell endocarp with elongated, lignified cells (Kapoor et al., 1995). After anthesis petals abscise and the fruit takes approximately 4 weeks to dry and dehisce. Capsules of *P.*

somniferum cv ‘persian white’ dry but do not dehisce; in this variety, pores only form occasionally, and seeds often need to be removed by breaking the pericarp.

2.2. *Eschscholzia californica* (Papaveraceae- Ranunculales)

Eschscholzia californica is an herb with compound, alternate or sometimes sub-opposite leaves. It is native to south-western North America. Phylogenetic analyses show that *Eschscholzia* together with the genera *Dendromecon* and *Hunnemania* are the three members of the subfamily Eschscholzioideae (Shannon, 2011), the putative sister group subfamily of Papaveroideae. *E. californica* possesses a chromosome number of 2n=12. Stable and transient transformation techniques have been standardized in this species (Park and Facchini, 2000; Wege et al., 2007). Seeds germinate in moist soil after a week. A week after germination the first true leaf expands. The species does not require vernalization to flower, and it does so 10 weeks after germination. *E. californica* produces a terminal flower associated with two cauline leaves with axillary inflorescence meristems (Becker et al., 2005); these also develop terminal flowers and associated axillary meristems resulting in compound cymose inflorescences with three orders of branching.

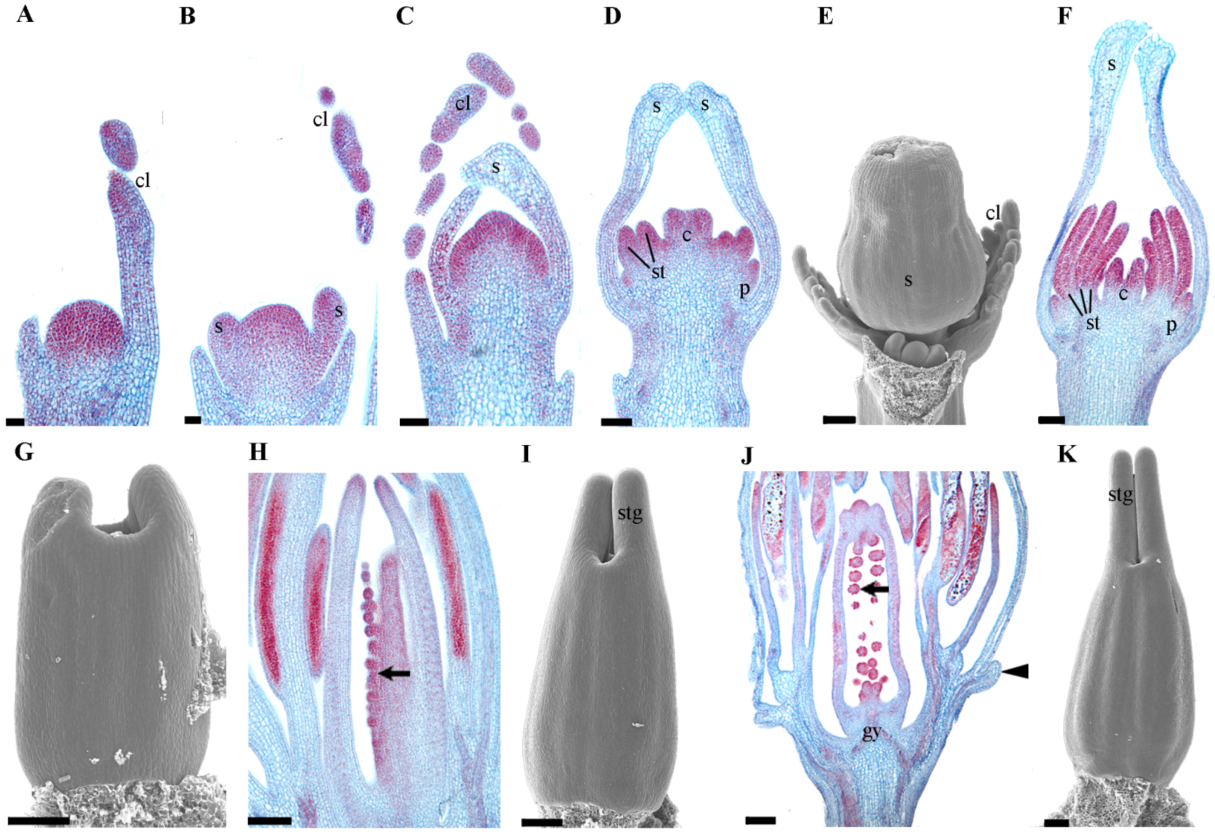

Figure 2.3. Flower development of *Eschscholzia californica*.
A, B Floral primordium before sepal initiation, subtended by two sub-opposite cauline leaves. **B.** Floral primordium during sepal initiation. . C-F. Successive stages of floral development showing sepal elongation and petal, stamen and carpel initiation. **G.** Young gynoecium on a short gynophores; the ovary is formed by 2 carpels, is unilocular and has parietal placentation. Arrow: ovules; arrowhead: floral rim. **cl,** cauline leaves; **s**, sepal or sepal primordium. A-C: 50 µm; D-K: 100 µm.

In *Eschscholzia californica* the floral primordium is elliptic as the two sepal primordia initiate on the median plane (Becker et al., 2005)(Figures 2.3A, B). Sepals develop from a ring primordium around the central floral dome and grow synchronously as a fused structure with two apical buldges and a deep constriction suggesting the congenital fusion of the two sepal primordia (Figures 2.3C, E) (Becker et al., 2005). When sepals start to cover the floral apex, two petal primordia initiate in a lateral position alternate with the sepals, and two other petals are almost simultaneously initiated opposite to the sepals (Figure 2.3C). Outer lateral petal primordia are narrower than inner medial petal primordia. Petals are usually retarded in their growth. The first four staminal primordia initiate between the petals; followed by the centripetal initiation of four more whorls of six stamens (Figure 2.3D) (Becker et al., 2005). Finally a circular gynoecium primordium initiates and, quickly differentiates into two elongating primordia (Figures 2.3D, F). The gynoecium, like in *P. somniferum*, stands on a short gynophore. The gynoecium is elongated and produces numerous ovules with parietal placentation (Figures 2.3G-K). It develops a short style and elongated stigmas with adaxial stigmatic surface (Figures 2.3G-K) (Becker et al., 2005). During floral organ elongation the hypanthium (or a fused receptacle or floral cup formed by the base of sepals, petals and stamens) grows above the level of the gynoecium, changing the early hypogynous flower to a perigynous flower during development. The level at which sepals, petals and stamens become free is marked by a sepal outgrowth (Figure 2.3J) called the "floral rim". This zone marks the abscission zone for all floral organs (except the gynoecium) which are shed during anthesis or after fruit set (Becker et al., 2005).

Fruits of *Eschscholzia californica* are longitudinally dehiscent capsules. These have not been anatomically described before (Figure 2.4). The fertilized carpels have an epidermis (exocarp) formed of thick isodiametrical cells, a mesocarp, formed by collenchyma (toward the outside) and large vacuolated parenchymatic cells (toward the inside) interspersed with vascular bundles (Figure 2.4). The endocarp is multi-layered with tangentially elongated cells. During fruit development three major changes occur (Figure 2.4): 1) The cells located outside of each vascular bundle become lignified leaving intercalary parenchymatic areas throughout the pericarp, 2) Lignification also occurs surrounding the medial vein of each carpel (facing each placenta) and defines the loculicidal dehiscence area and 3) The cells forming the endocarp enlarge radially before dehiscence. Fruit dehiscence occurs 3-4 weeks after the perianth withers.

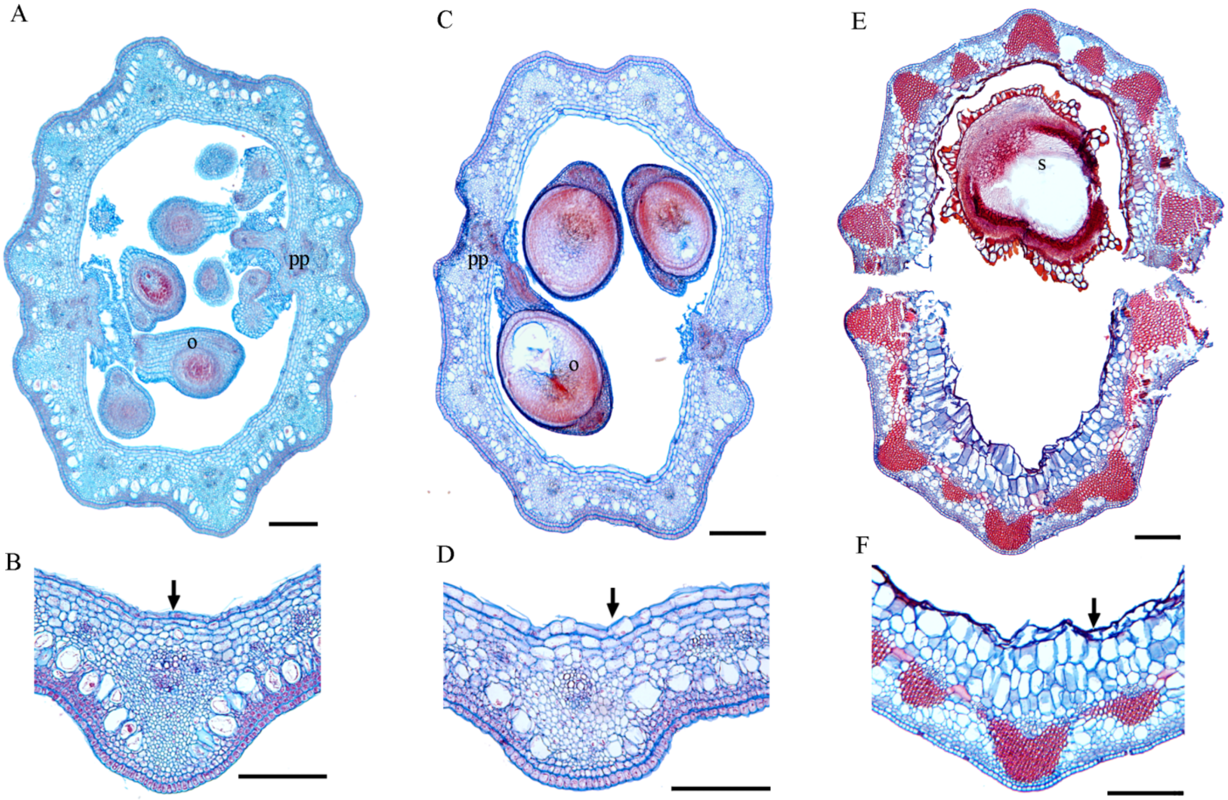

Figure 2.4. Ovary and fruit of *Eschscholzia californica*.
(**A)** 0.8cm ovary in anthesis **(B)** Detail of the carpel wall in B. **(C)** 1.5 cm fruit **(D)** Detail of the fruit wall in C. (**E)** 4 cm fruit **(F)** Detail of the fruit wall in E. Arrow: innermost layer of the endocarp A,B,D = 50µm; C = 70µm; E,F = 140 µm.

2.3. *Aquilegia coerulea* (Ranunculaceae- Ranunculales)

This species is a perennial herb with alternate, trifoliate leaves and deeply lobed leaflets. It is native to SW Montana to N Arizona and New Mexico, and belongs to a group of at least 13 species, from the New World, with nectar spurs in the petals (Whittall and Hodges, 2007). The genus belongs to the tribe Isopyrae (Tamura, 1993; Hodges, 1997). Species within *Aquilegia* have small genome sizes and can be crossed, which facilitates studies in genetics and genomics (Kramer and Hodges, 2010). The genome of *Aquilegia coerulea* has been sequenced and an extensive EST library is publicly available. Furthermore, transient transformation techniques have been standardized in *Aquilegia coerulea cv. origami,* a smaller variety with short life cycle and less vernalization time (Gould and Kramer, 2007). Seeds germinate in moist soil or vermiculite after one, sometimes two months. Two weeks after germination the first true leaf expands. Most species of *Aquilegia* require a period of exposure to cold temperature (vernalization) prior to flowering. This feature is relevant for this project in the context of evaluating what potential impact vernalization has in the activation of *FUL-like* genes (Danyluk et al., 2003; Trevaskis et al., 2003; Trevaskis et al., 2007; Preston and Kellogg, 2008). Vernalization has to be maintained for 20-25 days in the origami cultivar. Flowering occurs abundantly after 1-2 months after vernalization. Fruit set can take up to a month.

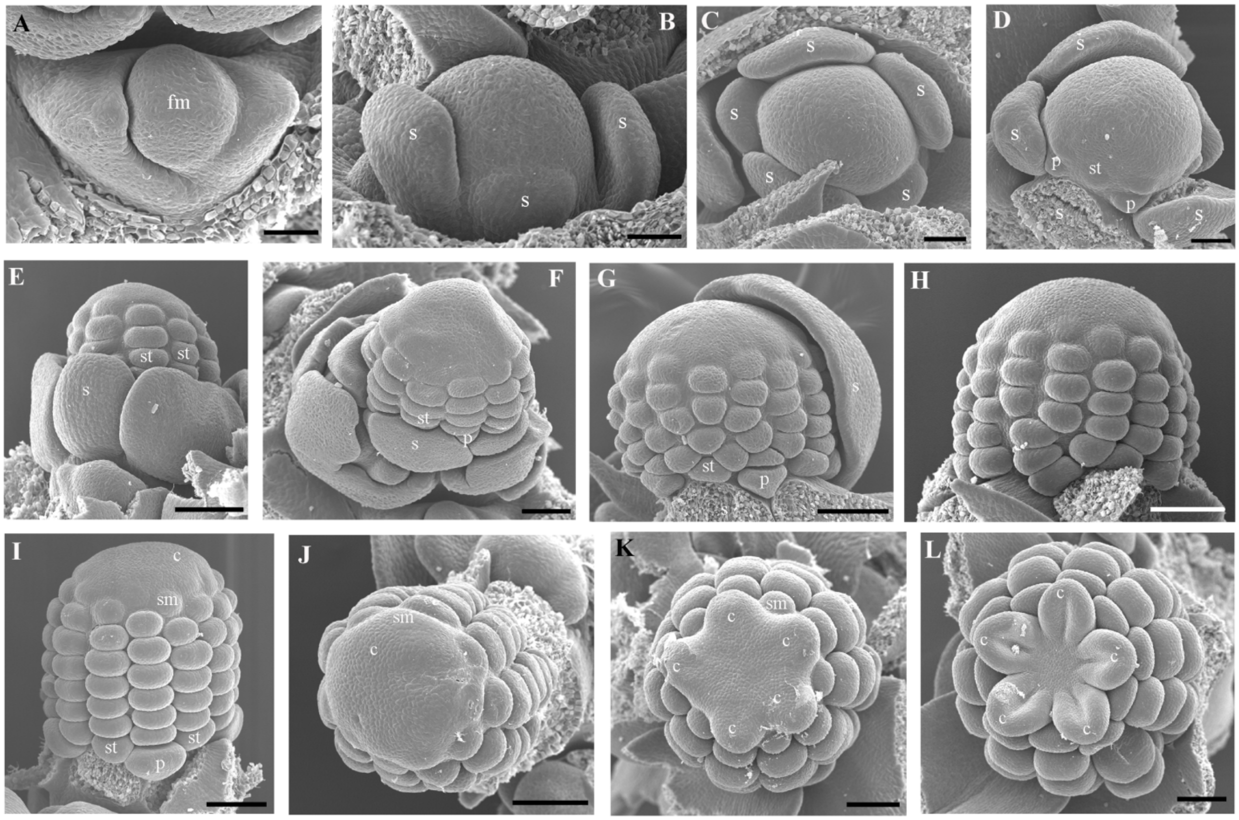

Figure 2.5. Flower development of *Aquilegia coerulea*.
c. carpel; fm, floral meristem; p, petal; s, sepal; sm, staminodia; st, stamen. **(A)** A = 25µm; B = 50µm; C-L = 100 µm.

Inflorescences of *A. coerulea* are cymes, with the oldest flower terminal and the younger ones in lateral positions. Inflorescences are terminal but additional inflorescences appear axillary to the rosette leaves after the shoot apex transitions to reproductive meristem. In general, the inflorescence consists of a main axis with 4 to 5 bracts. A terminal flower is produced above the uppermost bract. Second order shoots carrying bracteoles and terminal flowers develop axillary to the bracts, and in turn, third order shoots with bracteoles and terminal flowers can also develop from the axils of the second order bracteoles.

Aquilegia flowers are polysymmetric, with five free petaloid sepals, five free spurred petals, numerous stamens in 4-6 whorls and five staminodes that surround the five free carpels in the center of the flower (Tucker S.C., 2005; Ronse deCraene L., 2010). Flower development in *A. coerulea* follows a similar pattern to other *Aquilegia* species. In *A. coerulea* the young floral primordium is tangentially broad and radially narrow (Figure 2.5A). The lateral floral primordia are subtended by two bracteoles, whereas the terminal flower usually lacks bracteoles. The five sepals initiate in a helical pattern from two abaxial, to one adaxial and finally two lateral sepals with quincuncial aestivation (Figures 2.5B, C). The order of initiation results in a gradient of sepal size throughout development. Petal primordia initiate after sepal primordia in an alternisepalous position (Figure 2.5D). Petal primordia are narrow at their inception and quickly adopt a triangular shape (Figure 2.5D, G, I). The first whorl of five stamens initiates alternate with the petals (Figure 2.5D), simultaneous with a visible elongation of the floral apex. A second whorl of five stamens initiates alternate with the first staminal whorl and the sepals and opposite to the petals (Figure 2.5D), establishing the 10 staminal orthostichies (longitudinal rows of stamens). A total of 12 whorls (of five antepetalous or antesepalous stamens) form the first six rows, following the 10 established orthostichies (Figures 2.5 E to I). The seventh row is formed by two whorls of staminodia. Staminodia primordia (Figure 2.5I to K) are very similar to the stamen primordia, only a bit smaller. The floral apex ends in five antesepalous carpel primordia, which first grow flat and then become plicate leaving the center of the floral apex flat in the center of the apocarpous gynoecium (Figure 2.5L).

During late flower development the rectangular protective sepals become triangular in shape whereas the triangular petal primordia become flat at the apex and develop spurs. Although at anthesis both perianth organs are colored and ‘petaloid’ they have very different epidermal surfaces; whereas sepals have conical cells, petals have flat cells on their adaxial surface (Kramer et al., 2007).

After fertilization carpels become follicles that dry out and release the seeds through apical pores and sometimes longitudinal slits. The fruits consist of 5 follicles that remain attached at the base, with many seeds each.

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CHAPTER THREE

EXPRESSION AND FUNCTION OF *FUL-like* GENES IN *Eschscholzia californica* AND *Papaver somniferum* (Papaveraceae)*

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3.1. INTRODUCTION

The evolution of MADS-box genes has featured numerous duplications and losses, affecting a single species or entire clades of land plants (Purugganan et al., 1997; Alvarez-Buylla et al., 2000; Becker and Theissen, 2003; Hileman et al, 2006). These transcription factors play key roles in plant development, most notably floral organ identity (Bowman et al., 1991; 1993; Coen and Meyerowitz, 1991). It has been speculated that duplications and subsequent diversification of MADS-box genes may have been a factor in the evolution of morphological diversity in land plants and of angiosperms in particular (Alvarez-Buylla et al., 2000; Irish and Litt, 2005; Kaufmann et al., 2005; Hands et al., 2011). For example, within angiosperms, a cluster of duplications is correlated with the diversification of the core eudicots, a clade that encompasses the vast majority (roughly 75%) of extant flowering plants and that includes major model systems such as Arabidopsis, snapdragon, and tomato (Fig. 3.1). Among the gene lineages affected by this event are key regulators of floral organ identity: *APETALA1* (*AP1*, A-function), *APETALA3* (*AP3*, B-function), and *AGAMOUS* (*AG,* C-function) (Kramer et al., 1998; 2004; Litt and Irish, 2003; Stellari et al., 2004; Zahn et al., 2005; 2006). Comparative functional studies of *AP3* and *AG* orthologs in monocots, basal eudicots, and core eudicots suggest that fundamentally, the functions of these genes in petal, stamen and carpel identity (B and C function) were conserved before and after the core-eudicot duplication (Ambrose et al., 2000; Lamb and Irish, 2003; deMartino et al., 2006; Drea et al., 2007; Kramer et al., 2007; Dreni et al., 2011; Hands et al., 2011; Sharma et al., 2011). However, we lack data with which to evaluate the effect of the core-eudicot duplication on the function and evolution of the *APETALA1* lineage; furthermore, the data that do exist have led to doubts about the conservation of the A-function. In particular, we lack data from basal eudicot species that would allow us to evaluate whether or not the core-eudicot duplication resulted in changes in the function of core-eudicot *APETALA1*-lineage genes.

As a result of the duplication, core-eudicot species possess two types of *AP1* lineage genes, *euAP1* and *euFUL*, and species outside of the core eudicots have only one type of gene (*FUL-like* genes) (Litt and Irish, 2003; Preston and Kellogg, 2006; Shan et al., 2007; Litt and Kramer, 2010) (Fig. 3.1). FUL-like and euFUL proteins possess a six hydrophobic amino-acid motif (FUL-like motif) of unknown function near the C terminus (Litt and Irish, 2003; Vandenbussche et al., 2003; Shan et al., 2007; Liu et al., 2010) (Fig. 3.1). In contrast, the C-terminus of euAP1 proteins has an acidic transcription activation motif, which has been shown to activate transcription in a yeast system (Riechmann et al., 1996; Cho et al., 1999), and a farnesylation motif (CaaX), hypothesized to be important in mediating multi-protein complex formation (Yalovsky et al., 2000). The fact that new functional domains are found only in euAP1 proteins suggests that the functional capabilities they encode might be absent from FUL-like proteins.

Within the Brassicaceae an additional duplication occurred in the *euAP1* gene clade, producing the Arabidopsis paralogs *CAULIFLOWER* (*CAL*) and *APETALA1* (*AP1*) (Lowman and Purugganan, 1999; Alvarez-Buylla et al., 2006). *AP1* and *CAL* are expressed in floral meristems and in developing sepal and petal primordia (Mandel et al., 1992; Kempin et al., 1995; Ferrandiz et al., 2000; Blázquez et al., 2006); expression patterns of orthologs in other core eudicots are for the most part similar, although they may also include bracts and reproductive organs (e.g., Huijser et al., 1992; Hardenack et al., 1994; Berbel et al., 2001; Shchennikova et al., 2004; Sather and Golenberg, 2009). In strong Arabidopsis *ap1* mutants, sepals are converted to bract-like structures, petals are absent, and the bract-like organs of the first whorl subtend secondary flowers in the second whorl (tertiary flowers can also form) (Irish and Sussex, 1990; Mandel et al., 1992; Bowman et al., 1993). AP1 also forms a protein complex with SEUSS and LEUNIG which negatively regulates the C-function gene *AGAMOUS*, restricting it to the inner two whorls (Sridhar et al., 2006). *CAL* is redundant with *AP1* for the specification of floral meristem identity (Kempin et al., 1995; Ferrandiz et al., 2000). In other core eudicots, *euap1* mutants such as *squa* in snapdragon (Huijser et al., 1992), *mtpim* in *Medicago* (Benlloch et al., 2006) and *lemads-mc* in tomato (Vrebalov et al., 2002) lack proper floral meristem identity and produce a ramified inflorescence with fewer flowers. The flowers show leaf-like sepals, nevertheless petal identity is unaffected. This suggests that other *euAP1* orthologs function in proper floral meristem and sepal identity, features that are structurally and developmentally linked, but not in petal identity (Huijser et al., 1992; Theissen et al., 2000; Litt, 2007; Causier et al., 2010).

FRUITFULL (*FUL*) is expressed in cauline leaves and inflorescence meristems, and later in the carpel primordia and fruits (Gu et al., 1998). Arabidopsis has a second *euFUL* paralog, *AGL79*, however its sequence is highly divergent and it appears to be expressed in roots (Parenicová et al., 2004). In other core eudicots, expression of *euFUL* genes is broader, including leaves and cauline leaves (or bracts), inflorescence and floral meristems, all floral organs, fruits, and ovules (e.g., Hardenack et al., 1994; Immink et al., 1999; Wu et al., 2000; Muller et al., 2001; Calonje et al., 2004; Shchennikova et al., 2004; Sather and Golenberg, 2009). In Arabidopsis *ful* mutants, floral organ identity is not affected, however *FUL* is redundant with *AP1* and *CAL* in regulating floral meristem identity (Ferrandiz et al., 2000) and has a unique function in regulating cell differentiation during fruit development (Gu et al., 1998; Liljegren et al., 2000; Ferrandiz, 2002). *ful* mutants also have defects in cauline leaf development (Gu et al., 1998), and *FUL* has been shown to participate in regulating flowering time, axillary meristem activation, meristem determinacy, and plant longevity (Melzer et al., 2008). Functional studies of other *euFUL* genes are scarce. In petunia, silencing of *PFG* resulted in plants that remained vegetative (Immink et al., 1999). Over-expression of an *Antirrhinum euFUL* paralog, *DEFH28*, in Arabidopsis (Muller et al., 2001) and of *NtFUL* in tobacco (Smykal et al., 2007), resulted in fruits with defective lignification that failed to dehisce. These data suggest that other *euFUL* genes may have the same dual roles as *FUL*: an early role in promoting the transition to reproductive meristems and a late role in proper fruit development.

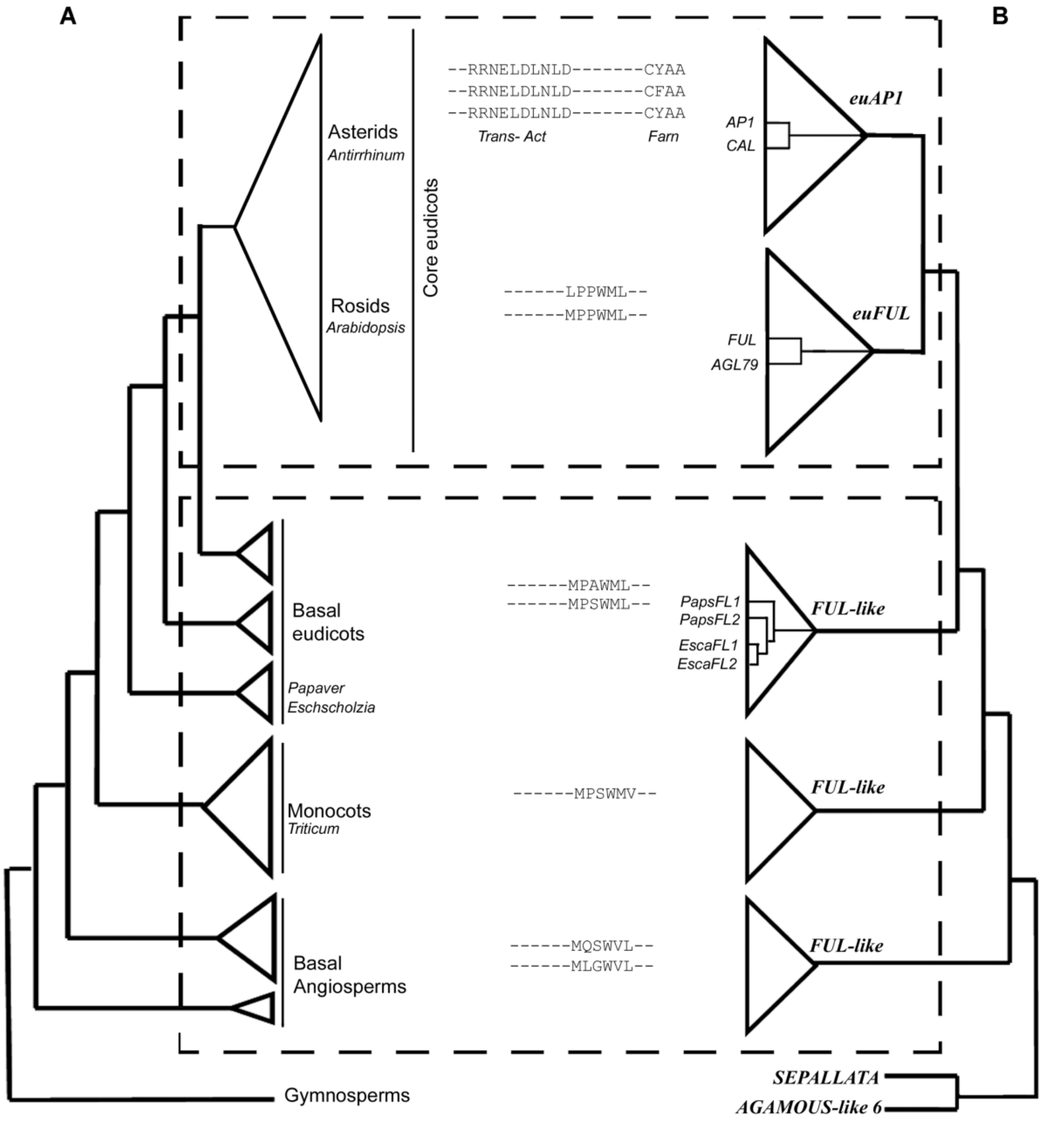

Figure 3.1. Simplified angiosperm phylogeny paired with the gene phylogeny of *AP1*/*FUL* homologs and their C-terminal motifs. Based on Litt and Irish (2003) and N. Pabón-Mora and A. Litt (unpublished data).
(A) Simplified angiosperm phylogeny showing the five major groups of flowering plants (basal angiosperms, monocots, basal eudicots, rosids, and asterids) and indicating the phylogenetic position of *Papaver* and *Eschscholzia*. The phylogenetic positions of *Arabidopsis*, *Antirrhinum* (snapdragon) and *Triticum* (wheat) are also shown. **(B)** Simplified *AP1/FUL* gene lineage tree, showing the core-eudicot gene duplication. The lower dotted box indicates the groups in which *FUL-like* genes are found, which includes all taxa outside of the core eudicots. The upper dotted box shows the *euAP1* and *euFUL* genes in core eudicots. Arabidopsis *AP1/FUL* homologs (*AP1*, *CAL*, *FUL*, *AGL79*) are shown in the gene tree, as are *PapsFL1*, *PapsFL2*, *EscaFL1*, and *EscaFL2*. In the center are the C-terminal protein motifs typical of the gene groups depicted in

(B): the euAP1 transcriptional activation (Trans-Act) and farnesylation (Farn) motifs and the FUL-like motif characteristic of euFUL and FUL-like proteins.

Non-core eudicot *FUL-like* genes, like *euFUL* genes, are broadly expressed in vegetative and reproductive tissues (e.g., Yu and Goh, 2000; Gocal et al., 2001; Chen et al., 2007; Preston and Kellogg, 2007, 2008; Wu et al., 2007; Danilevskaya et al., 2008); however, less is known about their function. Studies in cereals have shown that *FUL-like* genes are up-regulated in leaves and meristems in response to vernalization, and may promote inflorescence initiation (Murai et al., 2003; Trevaskis et al., 2003; 2007; Preston and Kellogg, 2007; 2008). Function is only known for *WAP1*, one of three *FUL-like* paralogs of wheat (*Triticum aestivum*), which is required for proper phase transition after vernalization (Murai et al., 2003). The data available suggest that *FUL-like* genes may be important in the transition from vegetative to reproductive meristems, but because of the lack of functional studies of *FUL-like* genes from species outside of the core eudicots, this remains an untested hypothesis.

Here we present data regarding the expression and function of *FUL-like* genes from *Eschscholzia californica* (California poppy) and *Papaver somniferum cv. Persian white* (opium poppy). Both taxa belong to the Papaveraceae, an early diverging family of basal eudicots; they are therefore evolutionary intermediates between the distantly related monocots and core eudicots. Importantly, both taxa are amenable to functional analysis using virus induced gene silencing (VIGS) (Hileman et al., 2005; Drea et al., 2007; Wege et al., 2007; Orashakova et al., 2009). Differences in floral morphology, in addition to the close relationship of these two species, make a comparison between them a robust platform from which to assess the role of *FUL-like* genes in basal eudicots. We test the following two alternative hypotheses regarding the functional evolution of this gene lineage: (1) Post-duplication *euAP1* and *euFUL* genes retained an ancestral role in mediating the floral transition and in floral meristem identity, but acquired new functions in perianth identity and fruit development, respectively. These new functions might be associated with changes in protein interactions and, in the euAP1 clade, with new sequence motifs. (2) Post-duplication *euFUL* and *euAP1* genes diverged functionally after the core-eudicot duplication event without acquiring new functions. According to this hypothesis, the “new” motifs of the euAP1 proteins do not confer novel functions. Rather, non-core eudicot FUL-like proteins encode the same functions as euAP1 and euFUL proteins combined.

3.2. RESULTS

California poppy and opium poppy are annual herbs that grow vegetatively as rosettes. Because both species have terminal flowers, the reproductive meristem initially has inflorescence character, forming cauline leaves subtending axillary buds. However, after producing 4-6 cauline leaves, the apical meristem becomes a terminal floral meristem. Whereas in opium poppy there is a single terminal flower and dormant axillary buds (only in the lowermost cauline leaves), in California poppy the reproductive axis develops into a multiple-flowered cymose inflorescence. Each cyme has three floral buds, one large terminal flower and two smaller lateral flowers. There are three orders of branching in this species. Branches or flowers may develop from buds on the main axis (first order), from buds on the first order branches (second order), and from buds on the second order branches (third order) (appendix 3.5.a; supp. Fig. 1A). Flowers are very similar in the two species, having two sepals, two alternate whorls of two petals, a large number of stamens, and two (in California poppy) to eight (in opium poppy) carpels. The two species differ in the morphology of the sepals and fruit. Opium poppy has free sepals and a superior ovary that develops into a capsule that releases the seeds though apical pores. In contrast, California poppy has fused sepals and a floral cup that surrounds the semi-inferior ovary, which becomes a longitudinally dehiscent capsule. In addition, the floral cup delimits the lower persistent versus the upper deciduous region of the sepals during anthesis. Developmental landmarks in inflorescence and flower morphology have been described for these species (Ronse De Craene and Smets, 1990; Becker et al., 2005; Drea et al., 2007).

3.2.a. Identification of opium poppy and California poppy *FUL-like* genes

Two *FUL-like* paralogs in opium poppy, *PapsFL1* and *PapsFL2* had been previously identified (Litt and Irish, 2003). The full-length coding sequences share 78% nucleotide identity and 60% amino acid identity. Using degenerate primers designed to amplify *AP1/FUL* genes (following Litt and Irish, 2003), we also identified two *FUL-like* genes in California poppy; *EscaFL1* and *EscaFL2*; these share 82% nucleotide identity and 80% amino acid identity. Opium poppy and California poppy *FUL-like* genes fall within the basal eudicot *FUL-like* gene clade consistent with the phylogenetic position of these species, although the duplications appear to be independent (Fig. 3.1B; Pabón-Mora and Litt unpublished data). Sequence analysis predicts that all the proteins from opium poppy and California poppy possess the conserved FUL-like C-terminal motif (appendix 3.5.b; supp. Fig. 2).

3.2.b. Expression of *FUL-like* genes in opium poppy

Following floral development stages defined by Drea et al. (2007), we evaluated *FUL-like* gene expression in floral organs at late preanthesis stages of development (stage P7, petal expansion initiated, and P8, petals fully expanded inside the sepals), and anthesis, as well as in fruits and leaves, using RT-PCR. Results (Fig. 3.2A) show that both *PapsFL1*and *PapsFL2* are broadly expressed before and at anthesis in all floral parts as well as in leaves and the fruit.

A detailed examination of expression during early floral development (Stages P0 - P6) was performed using in situ mRNA hybridization. Results show that *PapsFL1* is expressed in developing cauline leaves throughout development (Fig. 3.2B–D) but is absent from the vegetative meristem (data not shown). Expression is seen in the young floral meristem, especially in sepal primordia (Fig. 3.2B). During early development at stage P3 (petal, stamen and carpel primordia are visible), *PapsFL1* is expressed in all floral organ primordia (Fig. 3.2C) and in the floral pedicel, and this expression continues through stage P6 (Fig. 3.2D-E). During stage P6 *PapsFL1* expression is evident in the carpel wall (Fig. 3.2F) and the septa towards the tip of the carpel (data not shown). *PapsFL2* is detected in the vegetative meristem (Fig. 3.2G), and it is also expressed in the developing cauline leaves during flower development, similar to *PapsFL1* (Fig. 3.2H). Starting in the stage P3 young floral meristem, *PapsFL2* expression becomes localized to the sepals (Fig. 3.2H–J). It appears to be absent from petal, stamen, and carpel primordia during stages P4 (Fig. 3.2J) and P5 (data not shown) but it is detected again at P6 in the carpel wall (Fig. 3.2K). *PapsFL1* and *PapsFL2* show a common expression pattern in the fruit wall (Fig. 3.2L) and the ovules (Fig. 3.2M-N), particularly in the inner integument and the nucellus at anthesis. In addition, both copies are also expressed in the dormant axillary meristems subtended by the lowermost cauline leaves (Fig. 3.2O). Hybridization with sense *PapsFL1* and *PapsFL2* probes showed no signal (appendix 3.5.c; supp. Fig. 3). A *P. somniferum PISTILLATA* (*PI*) probe (Drea et al., 2007) was used as a positive control and we detected its expression as previously described (appendix 3.5.c; supp. Fig. 3).

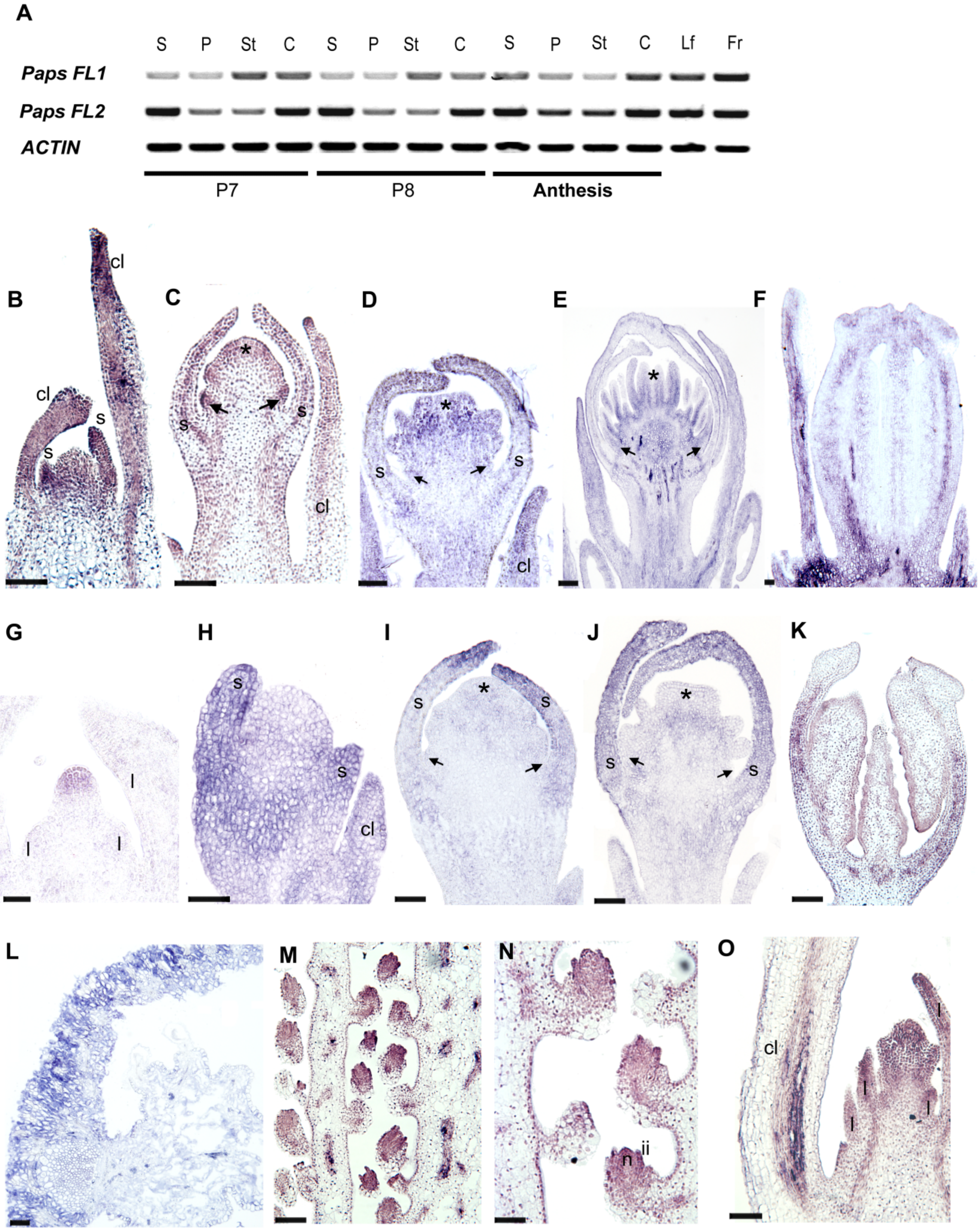
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Figure 3.2. Expression of *PapsFUL-like* genes at different developmental stages.
(A) RT-PCR results showing expression of *PapsFL1* and *PapsFL2* in dissected floral organs at different floral bud stages and in flowers at anthesis, as well as in fruits and leaves. Stages based on Drea et al. (2007). At bud stage P7 petal primordia have not elongated; at bud stage P8 petals are fully expanded inside the floral bud.
(B) to **(O)** In situ mRNA hybridization.
(B) to **(F)** Expression of *PapsFL1.*
(G) to **(K)** Expression of *PapsFL2.*
(**L**) to (**O**) Expression common to *PapsFL1* and *PapsFL2* (L, *PapsFL1* section; M-O, *PapsFL2* sections).
(B) and **(H)** Early floral meristem, sepal primordia are starting to differentiate.
(C) and **(I)** Floral bud with large sepals protecting the incipient petal, stamen and carpel primordia.
(D) and **(J)** Floral bud with overlapping sepals and fully differentiated petal, stamen and carpel primordia.
(E) Floral bud with clearly defined anther and filament.
(F) and (K) Longitudinal section of the carpel in a preanthesis floral bud.
(G) Shoot apical meristem before the transition to flowering.
(L) Cross section of the young fruit showing the fruit wall.
(M) and **(N)** Longitudinal section of the ovary showing placenta and ovules.
(O) Dormant axillary bud subtended by the lowermost cauline leaves.
c, carpel; cl, cauline leaf; fr, fruit; ii, inner integument; lf, leaf; l, leaf primordia; n, nucellus; p, petal; s, sepal; st, stamen. Arrows indicate petal primordia and asterisks indicate carpel primordia. Scale bars: B-E, H-J and O= 50µm, G= 70µm, F, K= 150µm, L= 100µm, M, N= 120 µm

3.2.c. Expression of *FUL-like* genes in California poppy

Using RT-PCR, we evaluated *FUL-like* gene expression in California poppy floral organs at the same late preanthesis developmental stages as in opium poppy (stage P7 and P8) and at anthesis, as well as in fruits and leaves. Results show that *EscaFL1* and *EscaFL2* are broadly expressed in all floral tissues at P7, P8, and anthesis, and both genes are expressed in fruits and leaves (Fig. 3.3A).

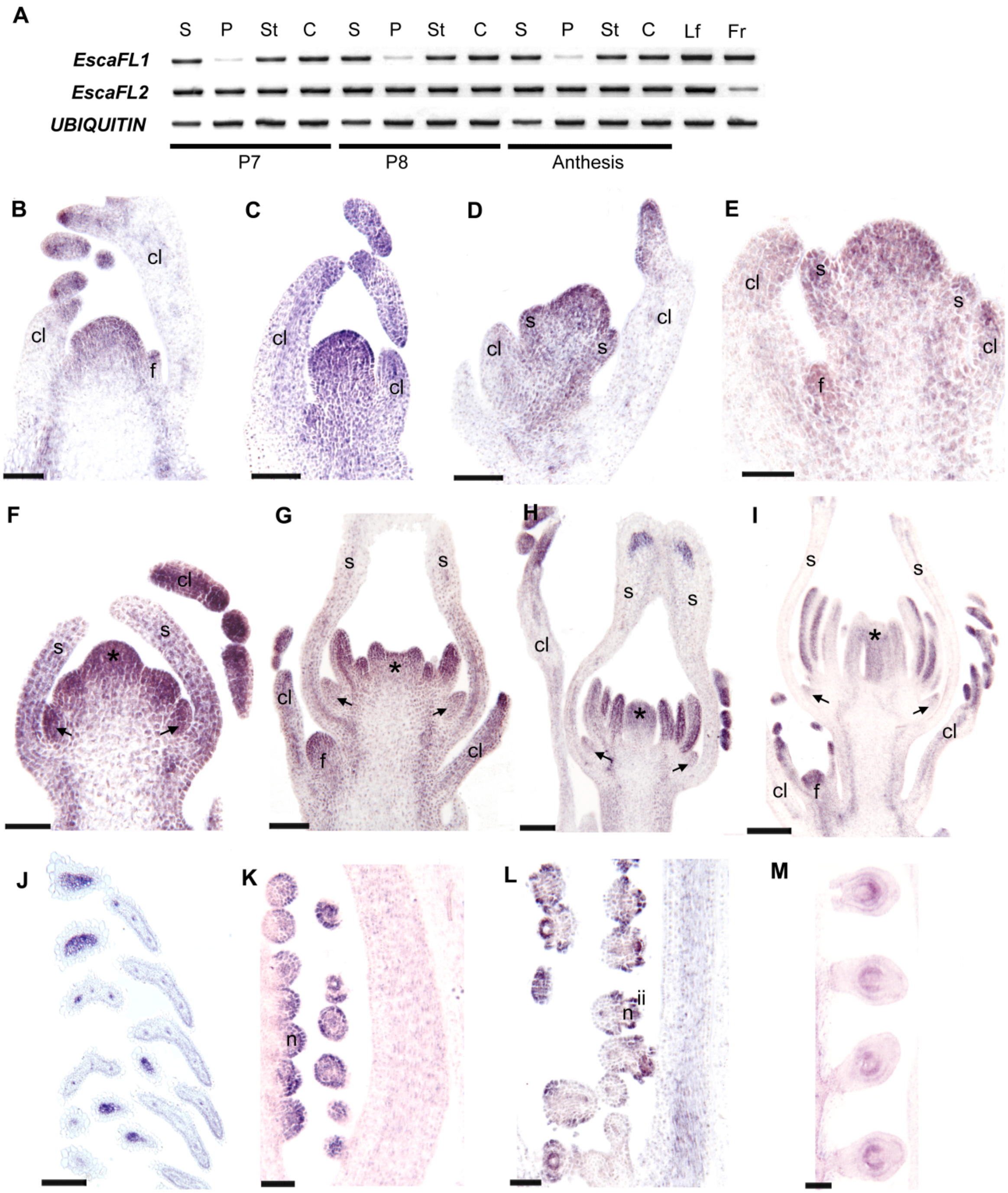
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Figure 3.3. Expression of *EscaFUL-like* genes at different developmental stages.

(A) RT-PCR results showing expression of *EscaFL1* and *EscaFL2* in floral buds (P7 and P8) and different parts of the flower at anthesis, as well as in leaves and fruits.
(B) to **(M)** In situ mRNA hybridization of *EscaFL1* and *EscaFL2*. Expression of *EscaFL1* and *EscaFL2* is identical. (B, E, F, G, H, J, K, L, and M, *EcFL1* sections; C, D, G, I, and N, *EscaFL2* sections).
(B) Inflorescence meristem protected by two highly dissected cauline leaves.
(C) and **(D)** Axillary inflorescence showing the terminal flower **(C)** before sepal initiation and **(D)** at the beginning of sepal initiation.
(E) Axillary inflorescence showing a terminal flower with sepal primordia and a floral meristem axillary to the cauline leaf on the left.
(F) Floral bud with elongating sepals enclosing petal, stamen and carpel primordia.
(G) Floral bud with fused sepals around petal, stamen, and carpel primordia.
(H) Floral bud with fully closed carpel wall.
(I) Floral bud with sporogenous tissue in the stamens. Sepals were forced open artificially in this sample.
(J) Tips of the highly dissected mature cauline leaves.
(K) to (M) Early stages of ovule development showing **(K)** the nucellus, **(L)** the initiation of the two integuments and **(M)** the fully formed bitegmic ovules.
c, carpel; cl, cauline leaf; f, axillary floral meristem; fr, fruit; ii, inner integument; im, inflorescence meristem; lf, leaf; n, nucellus; p, petal; s, sepal; st, stamen. Arrows indicate petal primordia and asterisks indicate carpel primordia. Scale bars: B-F= 60µm, G-J= 100µm , K-M= 50µm.

In situ mRNA hybridization during early floral development (Stages P0-P6) indicates that at the tissue level, expression patterns of *EscaFL1* and *EscaFL2* are identical. *EscaFUL-like* genes (both copies) are strongly expressed throughout the inflorescence meristem (Fig. 3.3B), in the provascular strands of the main reproductive axis (Fig. 3.3B), and in the primordia and growing tips of the cauline leaves (Fig. 3.3B–J). *EscaFUL-like* genes are also expressed throughout the young floral meristem before (Fig. 3.3C) and during early sepal differentiation (Fig. 3.3D-E). During stage P3, California poppy *FUL-like* genes continue to be expressed in the sepal primordia, and expression begins in petal, stamen, and carpel primordia (Fig. 3.3F). Expression in the sepals decreases basipetally during stage P4, particularly after the apical fusion of the sepals (Fig. 3.3G, H), and it remains only associated with the vasculature at the apex of the sepals (Fig. 3.3H). During stage P5, expression decreases in petals (Fig. 3.3G–I), but remains strong in stamens and carpels (Fig. 3.3H–I). During stage P6 the two genes are expressed in the carpel wall (Fig. 3.3L) and the developing ovule, particularly in the nucellus (Fig. 3.3K–M) and during the initiation of the two integuments (Fig. 3.3L). Hybridization with the sense *EscaFUL-like* probes showed no signal, and *EscaAG* (*Eschscholzia californica AG* ortholog) gene expression was used as a positive control (appendix 3.5.c; supp. Fig. 3).

3.2.d. Silencing of *PapsFUL-like* genes using TRV-VIGS

To investigate the function of *FUL-like* genes in basal eudicots we used VIGS, a transient post-transcriptional gene silencing mechanism that promotes degradation of target endogenous mRNAs in the plant (Dinesh-Kumar et al., 2003; Burch-Smith et al., 2004; Liu and Page, 2008). We used the bipartite Tobacco Rattle Virus, in which RNA1 (or TRV1) encodes the viral replicase and the RNA-dependent RNA polymerase, and RNA2 (or TRV2) encodes the coat protein and has a multiple cloning site for insertion of the endogenous target sequence. This approach has been successfully implemented in poppies (Hileman et al., 2005; Drea et al., 2007;Wege et al., 2007; Yellina et al., 2010) and other Ranunculales (Gould and Kramer, 2007; Kramer et al., 2007; Di Stilio et al., 2010). In order to specifically silence each *FUL-like* paralog in opium poppy we generated TRV2 constructs carrying a short fragment of *PapsFL1* or *PapsFL2* (appendix 3.5.b; supp. Fig. 2). In addition, the two constructs were mixed together to silence both copies simultaneously.

PapsFL1 – A total of 100 seedlings with 0-2 true leaves were infiltrated with TRV1 and TRV2-*PapsFL1* (appendix 3.5.b; supp. Fig. 2; appendix 3.5.g supp. Table 1). Seedlings were grown until flowering, and cauline leaves, sepals and fruits were screened using RT-PCR for the presence of TRV1 and TRV2 and for levels of *PapsFL1* transcript (appendix 3.5.d.; supp. Fig. 4A). Initially, based on RT-PCR results, plants were classified as unsilenced (those treated with TRV2-*PapsFL1* but showing no noticeable reduction in transcript) or showing mild, moderate, or strong down-regulation. However, similar mutant phenotypes were observed at all levels of down-regulation. Only the severity of the phenotypes and the number of flowers per plant displaying a specific phenotype increased as transcript levels were reduced. In 32% (n=27) of the treated plants there was down-regulation of *PapsFL1*, with no down-regulation of *PapsFL2*, demonstrating that the TRV2:*PapsFL1* construct was gene specific (appendix 3.5.d.; supp. Fig. 4A). The reduction of transcript abundance was confirmed using qRT-PCR (appendix 3.5.d.; supp. Fig. 4B), these experiments also suggest there may be an increase of *PapsFL2* transcript levels upon down-regulation of *PapsFL1*. Plants were examined for novel phenotypes throughout development. Whereas wild type opium poppy plants possess a single terminal flower (Fig. 3.4A), 78% of the plants with reduced levels of *PapsFL1* transcript (n=21) showed outgrowth of axillary branches after the terminal flower senesced and fruit development was initiated (Fig. 3.4B). The number of branches ranged from 2 to 6 (Fig. 3.4B). In 38% (n=8) of the branching *papsfl1* plants, the vegetative and cauline leaves showed apparent abnormal meristematic activity, resulting in broader, deeply lobed laminas accompanied by multiple mid-veins (Fig. 3.4D-E). In addition, in contrast to wild type cauline leaves, which decrease in size from the base to the apex of the inflorescence stem (Fig. 3.4A), the *papsfl1* cauline leaves were more uniform in size and larger than wild type (Fig. 3.4B). Analysis of epidermal cells showed no abnormalities compared to wild type (data not shown).

Figure 3.4. Inflorescence and cauline leaf phenotypes in opium poppy plants treated with TRV2- *PapsFL1* and TRV2- *PapsFL2* separately.
(A) Wild type opium poppy. (**B)** Plants down-regulated for *papsfl1*. (**C)** Plants down-regulated for *papsfl2*.
(D) to **(F)** leaf clearings of cauline leaves in **(D)** wild type opium poppy, (E) *papsfl1* plants and **(F)** *papsfl2* plants. Two cauline leaves are presented in each panel to illustrate variation of size and shape from larger more basal cauline leaves (left) to smaller more apical cauline leaves (right).
White arrows indicate axillary branches. Scale bars: A-C= 5cm; D, E, F= 1 cm.

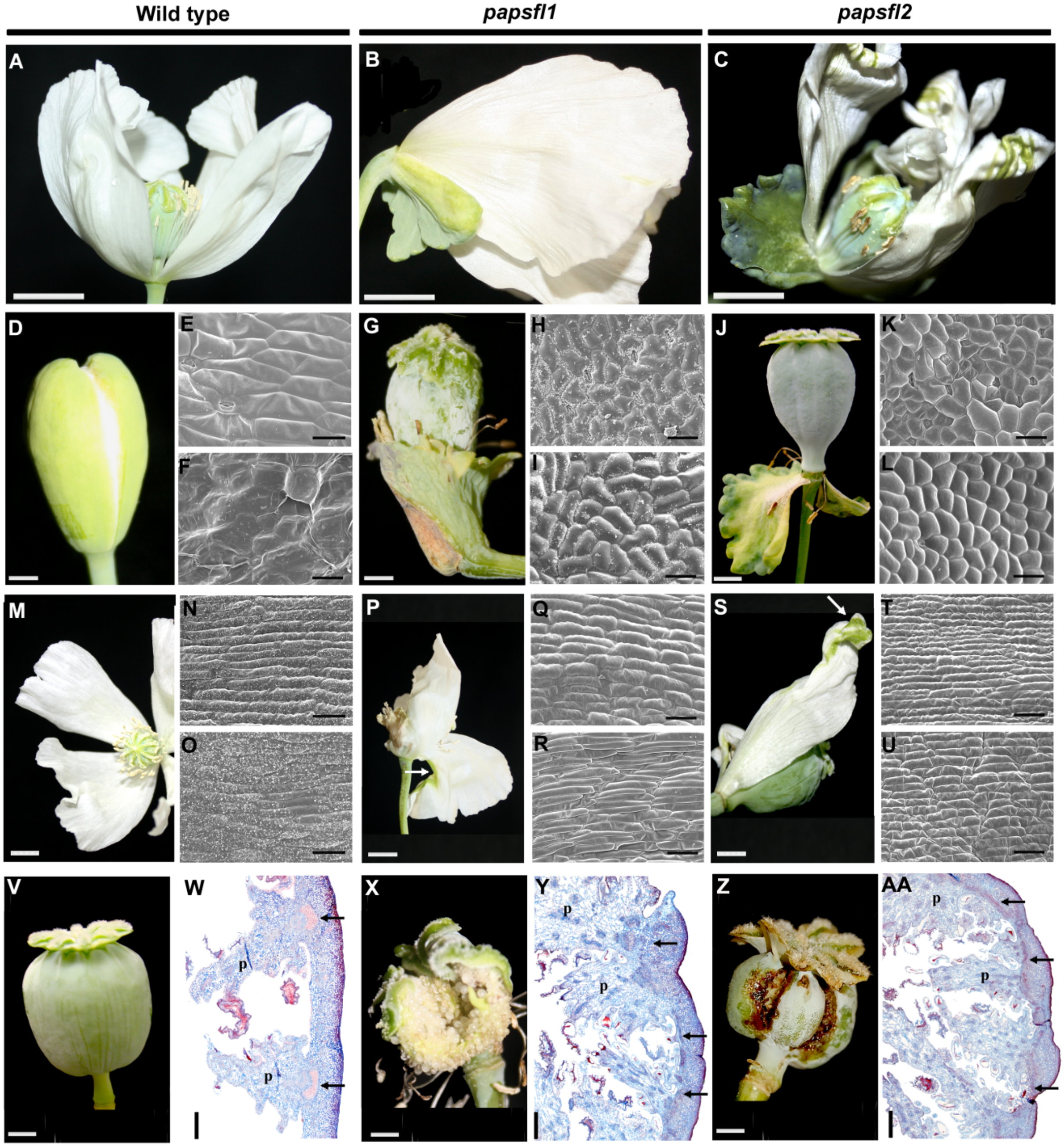


Figure 3.5. Floral phenotypes in opium poppy plants treated with TRV2-*PapsFL1* and TRV2-*PapsFL2* separately.

(A) Wild type opium poppy flower after deciduous sepals have fallen.
(B) and **(C)** Flowers showing persistent leaf-like sepals that remain attached to the base of the flower in **(B)** *papsfl1* plants and **(C)** *papsfl2* plants.
(D) Wild type sepals. **(E)** and **(F)** SEM of the **(E)** abaxial and **(F)** adaxial wild type sepal surfaces.

(G) persistent *papsfl1* leaf-like sepals during fruit development. **(H)** and **(I)** SEM of the **(H)** abaxial and **(I)** adaxial leaf-like *papsfl1* sepal surface.
(J) persistent *papsfl2* leaf-like sepals during fruit development. **(K)** and (**L)** SEM of the **(K)** abaxial and **(L)** adaxial leaf-like *papsfl2* sepal surface.
(M) Wild type opium poppy petals. **(N)** and **(O)** SEM of the **(N)** abaxial and **(O)** adaxial wild type petal surfaces.
(P) *papsfl1* flower showing small green patches on the abaxial surface of the outer petals. **(Q)** and **(R)** SEM of the petaloid **(Q)** abaxial and **(R)** adaxial *papsfl1* petal surfaces.
(S) *papsfl2* flowers showing small green areas in the distal portion of the petal. **(T)** and **(U)** SEM of the petaloid **(T)** abaxial and **(U)** adaxial *papsfl2* petal surfaces.
(V) Wild type opium poppy fruit (poricidal capsule formed by eight fused carpels).**(W)** Cross section of the Wt fruit showing main vascular bundles of each placenta.
(X) *papsfl1* fruit. **(Y)** Cross section of *papsfl1* fruits **(Z)** *papsfl2* fruit. **(AA)** Cross section of *papsfl2* fruits. P, placenta; black arrows point to vascular traces in the pericarp; white arrows point to green patches on the petals. Scale bars: A-C= 1.5 cm; D,G,J,P,V,X,Z= 0.5 cm; E,F,H,I,K,L,N,O,Q,R,T,U, = 40µm; M,S= 0.7 cm; W,Y, AA= 500 µm

In 47% (n=10) of the branching *papsfl1* plants, the sepals of one to three flowers per plant exhibited apparent homeotic transformation to leaf-like organs. They were characterized by lobed margins and a waxy cuticle, and remained attached to the base of the flower after anthesis and during fruit development (Fig. 3.5B and 3.5G). This is in contrast to the early deciduous sepals of wild type flowers (Fig. 3.5A), which lack a waxy cuticle and have entire margins (Fig. 3.5D). These leaf-like organs furthermore exhibited typical leaf cell types (Fig. 3.5H and 3.5I), in contrast to the cell types characteristic of sepals (Fig. 3.5E and 3.5F).

In general, *papsfl1* plants did not show defects in petal identity (Fig. 3.5B, Q and R) with the exception of occasional small green patches (Fig. 3.5P) the epidermis of which exhibited cells that appeared to be leaf- or carpel-like (data not shown). For the most part, the petal laminas exhibited the elongated rectangular cells typical of wild type petal epidermis (Fig. 3.5N and O). The identity of stamens and carpels was not affected in VIGS-treated plants (appendix 3.5.g.; supp. Table 1). However, 23% (n=5) of the *papsfl1* plants exhibited carpel defects, such as asymmetrical elongation of the carpel wall resulting in bending of the carpel and the fruit, and premature rupture of the fruits before full seed maturation (Fig. 3.5V and X). These fruits had lignification and placentation defects and irregularly thickened pericarps (Fig. 3.5W and Y). Nevertheless, epidermal features of the fruit are unchanged (data not shown). None of these abnormal phenotypes were observed in plants treated identically but transformed with an empty TRV2 vector lacking the target sequence (appendix 3.5.g; supp. Table 1; data not shown).

***PapsFL2* -** We infiltrated 80 seedlings of opium poppy with TRV1 and TRV2-*PapsFL2*. Seedlings were grown and screened as for *PapsFL1*. In 19% (n=13) of the plants there was down-regulation of *PapsFL2* with no change or even an increase of *PapsFL1* transcript abundance (appendix 3.5.d.; supp. Fig. 4C and 4D). As in *papsfl1* plants, the extent of down-regulation was somewhat correlated with the severity of the mutant phenotype, but the types of defects were the same regardless of the degree of down-regulation. Phenotypes were similar to those observed for *PapsFL1*, including branched inflorescences (n=10) (Fig. 3.4C; appendix 3.5.g.; supp. Table 1), abnormally broad, lobed cauline leaves (n=5) (Fig. 3.4C and F), partial or total homeotic conversion of deciduous sepals to persistent leaf-like organs with leaf-like epidermal cells (n=7) (Fig. 3.5C and J) and defects in carpel and fruit development (n=4)(Fig. 3.5Z) including anatomical defects and premature fruit rupture similar to what was observed in *papsfl1* plants (Fig. 3.5AA). Petals were mostly unaffected (Fig. 3.5S–U), however, they occasionally developed distal abaxial green areas (Fig. 3.5C and S) with leaf- or carpel-like epidermal cells similar to what was seen in *papsfl1* plants (data not shown).

PapsFL1-FL2 – A total of 108 seedlings of opium poppy were infiltrated with TRV1, TRV2-*PapsFL1*, and TRV2-*PapsFL2* in a 1:1:1 ratio. Seedlings were grown and screened as before. Some plants showed down-regulation of only one copy, as expected due to the heterogeneous systemic spread of the two different vectors. Plants showing down-regulation of only one gene were not studied further. 13.8% (n=15) of the plants showed down-regulation of both *PapsFL1* and *PapsFL2* (appendix 3.5.d.; supp Fig. 4E and 4F) (henceforth referred to as *papsfl1-fl2*). Of the double down-regulated plants, 11% (n=12) showed a delay in reproductive transition as evidenced by bolting that was 1-2 weeks later than wild type plants. To confirm this observation, the number of total leaves was counted. Untreated wild type plants (n=12) produced an average of 11 ± 0.20 leaves, and treated plants that showed no down-regulation produced an average of 11± 0.60 leaves (n= 8) before producing an inflorescence axis; in contrast *papsfl1-fl2* plants (n=12) produced 18± 0.56 leaves ($p<0.001$). Morphological phenotypes observed for *papsfl1-fl2* plants were similar to those documented for down-regulation of each gene independently, and included branching in the inflorescence (n=7), overgrowth and shape defects in cauline leaves (n=5), leaf-like sepals (n=5), and defects in carpel and fruit development (n=5) (data not shown). In addition, a novel morphological phenotype emerged when both copies were simultaneously down-regulated. Double *papsfl1-fl2* plants showed large patches of green tissue on the abaxial and adaxial surfaces of the two outer petals, in some cases occupying up to 70% of the petal area (Fig. 3.6A–D). SEM examination showed a loss of wild type petal cell identity on both the abaxial (Fig. 3.6E and H) and adaxial (Fig. 3.6F and I) surfaces, replaced by apparent leaf- or carpel-like cell identity (Fig. 3.6J–O). To test whether this phenotype was associated with ectopic expression of C-function genes, we evaluated transcript levels of *PapsAG1* and *PapsAG2* in the green petals and carpels of *papsfl1-fl2*. *PapsAG1* and *2* have been shown to specify stamen and carpel identity in opium poppy; down-regulation of both *PapsAG* homologs concurrently resulted in a homeotic transformation of the third and fourth whorl androecium and gynoecium into petaloid and sepaloid and organs respectively (Hands et al., 2011). However, the leaf- or carpel-like tissue in the *papsfl1-fl2* petals was not associated with over-expression of *PapsAG1 or PapsAG2* when compared to the wild type petal (data not shown). Interestingly the inner two petals in down-regulated flowers always showed wild type morphology and epidermis.

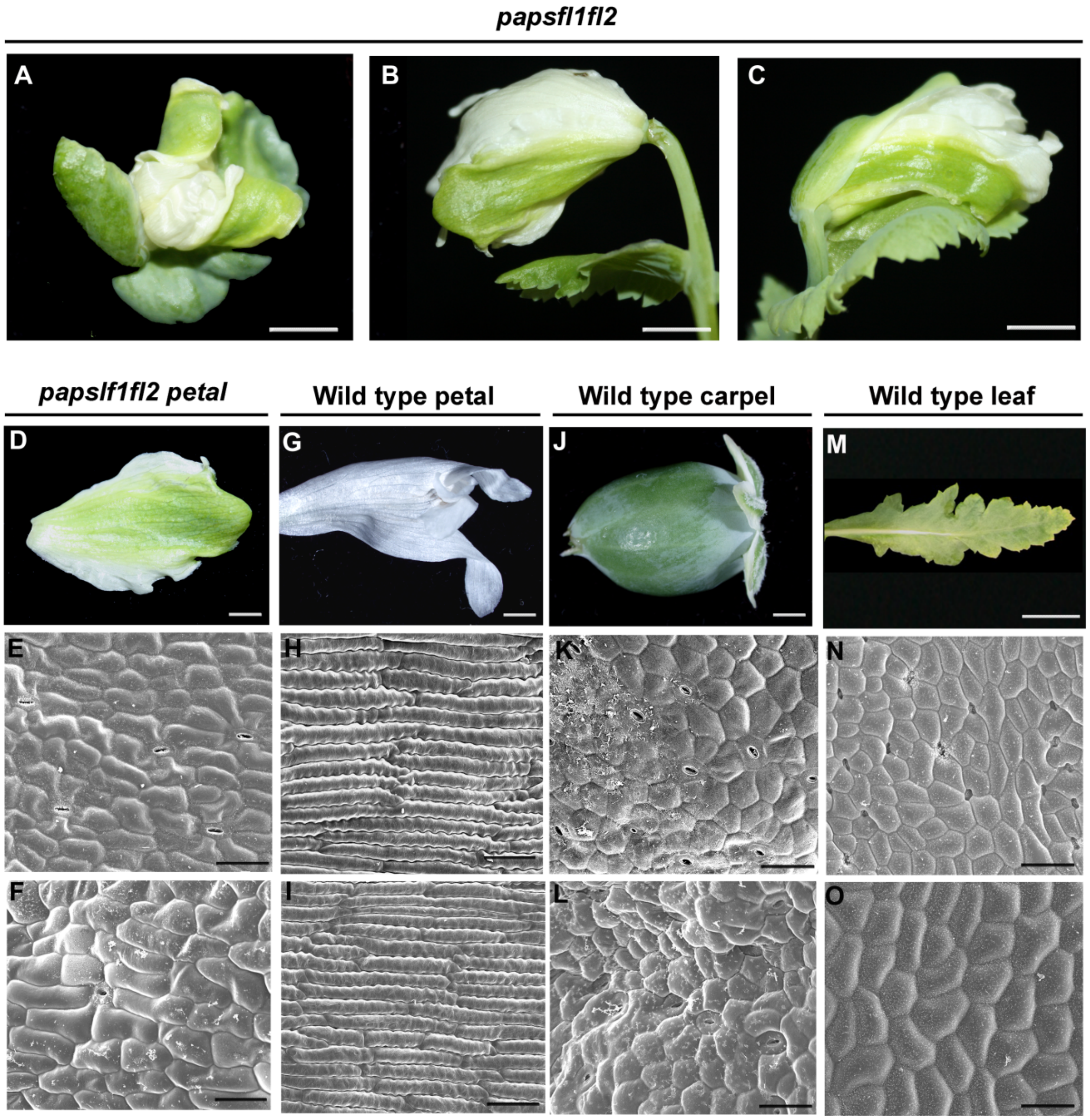


Figure 3.6. Floral phenotypes in opium poppy plants treated with TRV2-*PapsFL1* and TRV2-*PapsFL2* simultaneously.

(A) to **(C)** Double down-regulated plants showing large green areas in the two outer petals in opium poppy. **(A)** Front view. **(B, C)**. Side views.
(D) Dissected *papsfl1-papsfl2* green petal.
(E) and **(F)** SEM of the **(E)** abaxial and **(F)** adaxial *papsfl1-papsfl2* petal surface.
(G) Dissected wild type petal.
(H) and **(I)** SEM of the **(H)** abaxial and **(I)** adaxial wild type petal surface.
(J) Dissected wild type carpel.
(K) and **(L)** SEM of the **(K)** abaxial and **(L)** adaxial surface of young wild type carpel. **(M)** Dissected wild type leaf.
(N) and **(O)** SEM of the **(N)** abaxial and **(O)** adaxial surface of wild type leaf.
Scale Bars: A-C= 0.75 cm; D,G,J, M = 0.5 cm; E, F, H, I, N, O= 50μm; K,L= 30μm.

3.2.e. Interactions among opium poppy FUL-like proteins

Since the single *papsfl1* and *papsfl2* down-regulated plants showed identical phenotypes, we wanted to test whether PapsFL1 and PapsFL2 could potentially interact to function as a dimer. We used a yeast 2 hybrid system to test for potential homo- and heterodimerization of PapsFL1 and PapsFL2 (appendix 3.5.e; supp. Fig. 5). Interaction between PapsFL1 and PapsFL2 is strong in both directions. PapsFL1 proteins form weak homodimers in the yeast system, but PapsFL2 proteins do not (appendix 3.5.e; supp. Fig. 5).

3.2.f. Silencing of *EscaFUL-like* genes using TRV-VIGS

To silence California poppy (*Esca*) *FUL-like* genes we designed two different vectors, TRV2:*EscaFL1* and TRV2:*EscaFL2*, carrying gene-specific inserts (appendix 3.5.b; supp. Fig. 2). Sixty seedlings with 0-2 true leaves were infiltrated with TRV1 and TRV2:*EscaFL1* and 60 with TRV1 and TRV2:*EscaFL2.* Seedlings were grown and screened as for the opium poppy experiments. A total of 40 plants showing abnormal phenotypes and 17 with wild type appearance were screened. All 40 plants with abnormalities showed reduced transcript levels. However independent of the TRV2 vector used, both *EscaFL1* and *EscaFL2* showed some degree of down-regulation (appendix 3.5.d; supp. Fig. 4G and 4H) (henceforth referred to as *escafl1-fl2*).

Table 3.1. Comparisons between number of first and second order branches in California poppy wild type plants and *escafl1-fl2* plants.

	Wild type	***escafl1-fl2* plants**
Number of first order branches	5.4 ± 0.62	9.16 ± 0.5
Number of second order branches	3.16 ± 0.66	9.11 ± 1.04
ANOVA (α: 0.005) first order branching p value <0.001		
ANOVA (α: 0.005) second order branching p value <0.001		

Values are mean ± standard error. n= 18 for both groups.

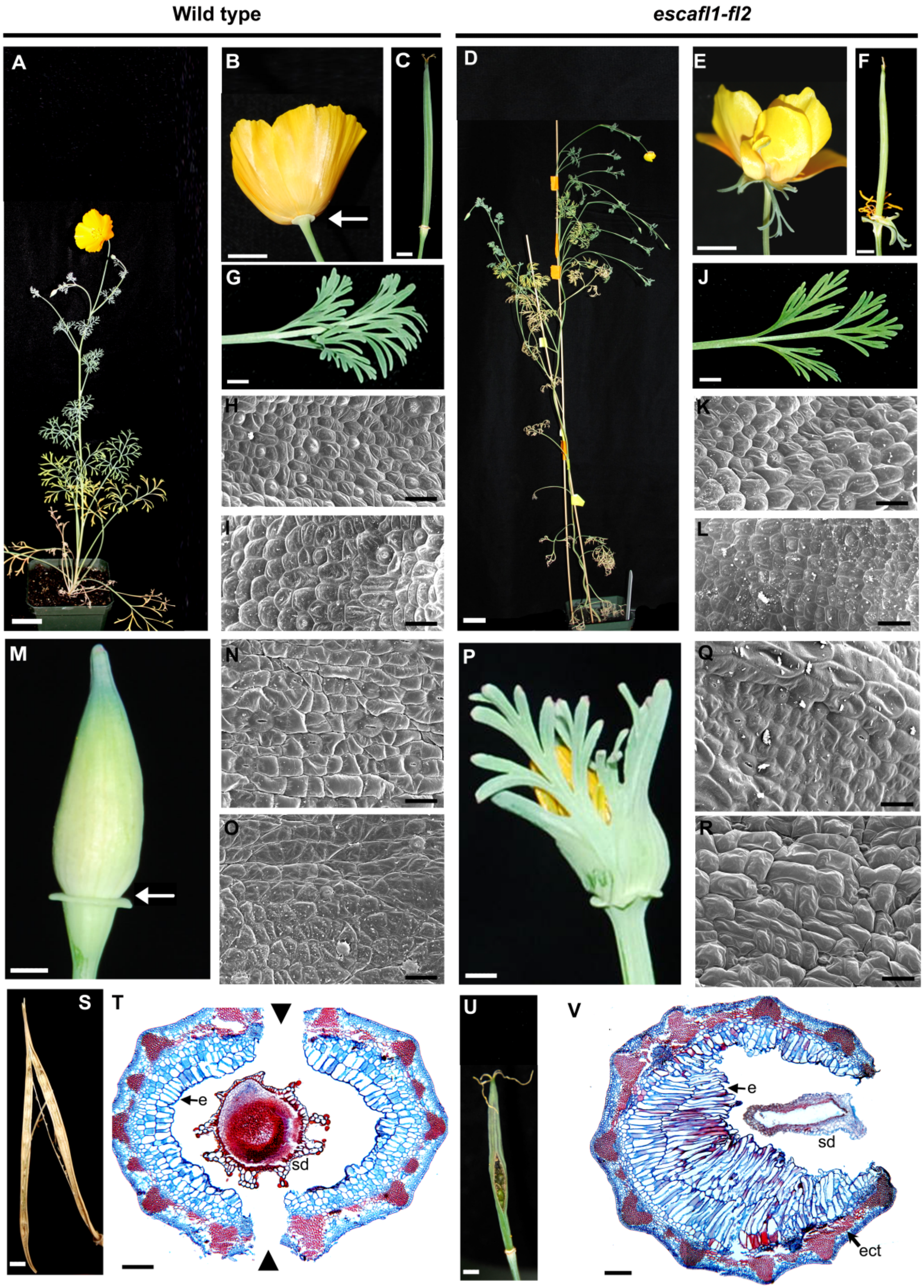

Wild type
escafl1-fl2
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
e
sd
U
V
e
sd
ect

Figure 3.7. Floral phenotypes in California poppy plants treated with TRV2-*EscaFL1* and TRV2- *EscaFL2.*

(A) to **(C)** Wild type California poppy plant. **(B)** flower showing the persistent floral cup after sepal abscission, and **(C)** fruit.
(D) to **(F)** Down-regulated *escafl1-fl2* plant showing **(D)** increased branching compared to wild type and **(E)** and **(F)** leaf-like organs replacing the sepals and persisting **(E)** after anthesis and **(F)** during fruit development
(G) Wild type cauline leaf
(H) to **(I)** SEM of the **(H)** abaxial and **(I)** adaxial wild type cauline leaf surface.
(J) Cauline leaves in *escafl1-fl2* plants
(K) and **(L)** SEM of the **(K)** abaxial and **(L)** adaxial *escafl1-fl2* cauline leaf surface.
(M) Wild type fused sepals before anthesis
(N) and **(O)** SEM of the **(N)** abaxial and **(O)** adaxial wild type sepal surface.
(**P**) Homeotic transformation of sepals into leaf-like organs
(Q) and **(R)** SEM of the **(Q)** abaxial and **(R)** adaxial leaf-like *escafl1-fl2* sepal surface. **(S)** Dehisced wild type fruit. **(T)** Cross section of the wild type fruit before dehiscence showing the ring of lignified tissue and the two dehiscence zones.
(U) *escafl1-fl2* fruit showing premature rupture of the fruit wall and exposure of the immature seeds. **(V)** Cross section of the *escafl1-fl2* fruit showing the lignified ring interrupted by a thinner weaker section of the pericarp through which the fruit ruptures.
White arrows point to the abscission zone between the floral cup and the deciduous portion of the sepals. Arrowheads point to the dehiscence zones in the fruit. e, endocarp; ect, ectopic lignification; sd, seed. Scale Bars: A,C= 3cm; B,D,E,F,I,R,T= 0.5 cm; L,O= 0.3 cm; G,H,J,K,M,N,P,Q= 20μm; S,U= 0.1mm.

The phenotypes of *escafl1-fl2* plants were similar to those observed in *papsfl1* and *papsfl2* plants. As with poppy, specific phenotypes were not correlated with degree of down-regulation of *FUL-like* transcripts, but the severity of the phenotypes was somewhat correlated. *escafl1-fl2* plants showed a significant increase in the number of first and second order branches (p<0.001 for both), compared to wild type (Fig. 3.7A, 3.7D, appendix 3.5.a.; supp Fig 1, Table 3.1). First order branches also grew longer, developing more leaves and associated axillary floral meristems. In contrast to opium poppy, cauline leaves in the down-regulated California poppy plants were identical in size, shape and epidermal features to wild type (Fig. 3.7G–L). In addition, although *escafl1-fl2* plants grew taller and had more branches, there was no observed difference in flowering time when compared to the wild type plants growing side by side. In 100% (n= 40) of the *escafl1-fl2* branched plants, one or both sepals of 1 to 4 flowers per inflorescence were partially or completely transformed into persistent leafy organs that remained attached to the base of the flower (Fig. 3.7E and P) and fruit (Fig. 3.7F) unlike the wild type deciduous sepals (Fig. 3.7B, C and M). Complete transformation was associated with the loss of the floral cup (appendix 3.5.f.; supp. Fig. 3.6J– L). These un-fused organs possessed highly dissected margins characteristic of leaves, instead of the normal smooth-margined, fused calyx found in wild type plants (Fig. 3.7E, M, P; appendix 3.5.f.; supp. Fig. 6). They also have epidermal features similar to those of leaves (Fig. 3.7Q and R), in contrast to those of wild type sepals (Fig. 3.7N and O). *escafl1-fl2* plants did not show defects in petal, stamen or carpel identity (Fig. 3.7E, appendix 3.5.g.; supp. Table 1). In addition, because leaf-like sepals were persistent at the base of the flower, in some *escafl1-fl2* plants the other floral organs did not expand properly and remained trapped inside the leafy sepals (appendix 3.5.f.; supp. Fig. 6).

Down-regulation of the two *EscaFL* paralogs in California poppy produced fruit defects similar to those seen in opium poppy. In 10% (n=4) of the *escafl1-fl2* plants we observed shorter fruits and premature rupture of the carpel wall (Fig. 3.7S–V). This may be the result of ectopic lignification of the pericarp and irregular growth of the innermost layer of the fruit (i.e. the inner endocarp) (Fig. 3.7V). In the wild type fruit, lignification is only associated with vascular traces, and the cells of the innermost layer of the fruit are slightly larger than cells in adjacent layers (Fig. 3.7T). In the down-regulated plants, lignification is also seen between vascular bundles, and the cells of the inner layers of the pericarp are dramatically elongated (Fig. 3.7V).

3.3. DISCUSSION

3.3.a. Expression domain of basal eudicot *FUL-like* genes includes the domains of both *euFUL* and *euAP1* homologs.

In general, opium poppy and California poppy *FUL-like* genes have broad expression patterns throughout plant development (Fig. 3.2 and 3.3). After germination, *FUL-like* genes in both species are expressed in leaves. Once the plant has transitioned to reproduction, transcripts are found throughout the inflorescence and floral meristems, in the growing tips of cauline leaves, and in the axillary meristems. Expression of *FUL-like* genes is maintained during the early differentiation of all floral organs (stages P3-P4), with the exception of *PapsFL2* expression, which is restricted to sepals. At later stages (stages P5-P6), *EscaFL1* and *EscaFL2* expression becomes localized to stamens and carpels. Both opium poppy and California poppy *FUL-like* genes are expressed in the carpel wall and ovules, and during fruit development.

These expression patterns are consistent with those reported for *FUL*-like genes from basal angiosperms, monocots, and basal eudicots, as well as those for core-eudicot *euFUL* genes. Expression of these genes tends to be broad, reported often in leaves, bracts or cauline leaves, inflorescence and floral meristems, most or all floral organs, fruits, and ovules (Reviewed in Fornara et al., 2004; Litt, 2007; Chen et al., 2008; Danilevskaya et al., 2008; Preston and Kellogg, 2008; Sather and Golenberg, 2009). In contrast, the expression patterns of *euAP1* genes are very different; *euAP1* expression is mainly restricted to floral meristems and floral organs, particularly sepals and petals, with expression also reported in some species in bracts, carpels, and ovules (e.g. Bowman et al., 1993; Hardenack et al., 1994; Ferrandiz et al., 2000; Berbel et al., 2001; Shchennikova et al., 2004; Sather and Golenberg, 2009). Our data and previously published data indicate that expression of *FUL-like* genes is present at all the developmental stages and in all the spatial domains that have been reported for the *euFUL* and *euAP1* genes. Thus, the pre-duplication expression domain of *AP1/FUL* genes includes a broad range of stages and organs; after the core-eudicot duplication the *euFUL* genes maintained this broad expression, whereas *euAP1* expression became restricted to a narrower domain consisting mainly of the floral meristem, sepals and petals. This differential repression (or the lack of up-regulation) in vegetative organs and reproductive floral organs may account for functional differences of *euAP1* genes when compared to the *euFUL* and *FUL-like* genes.

3.3.b. *FUL-like* genes function pleiotropically during plant development.

We have shown that down-regulation of *FUL-like* genes in the two Papaveraceae species results in changes in inflorescence architecture and defects in floral meristem identity as shown by the homeotic transformation of sepals into leaf-like organs (Fig. 3.5D, G, and J; 3.7M and P). In addition, fruit development is abnormal and fruits rupture prematurely (Fig. 3.5V–AA; 3.7S–V). These phenotypes are consistent with functions commonly described for other *AP1/FUL* homologs. Arabidopsis *AP1*, *CAL*, and *FUL*, and *euAP1* orthologs in other species such as snapdragon, tomato, pea, and Medicago have been shown to be involved redundantly in the determination of proper floral meristem identity (Huijser et al., 1992; Berbel et al., 2001; Taylor et al., 2002; Vrebalov et al., 2002; Benlloch et al., 2006). However, because redundancy of *euAP1* genes with *euFUL* genes has not been investigated except in Arabidopsis, it is unclear whether proper floral meristem identity is a common role for other *euFUL* genes. In addition, the fact that homeotic conversion of sepals into leaf like organs in *Eschscholzia* results in the loss of the floral cup suggests that the differentiation of this structure is dependent on correct floral meristem and sepal identity. *FUL* plays a role in maintaining proper carpel wall growth during fruit development in Arabidopsis, and experiments over-expressing the *euFUL* gene *DEFH28* from snapdragon in Arabidopsis (Muller et al., 2001), and silencing *euFUL* copies such as *VmTR4* from bilberry (Jaakola et al., 2010) and *SlMBP7* from tomato (R. Meyer, N. Pabón-Mora and A. Litt unpublished data), suggest that other *euFUL* genes also play a role in fruit development. We have shown that *FUL-like* genes in Papaveraceae also play a role in proper fruit development similar to that of *FUL* and other *euFUL* genes, including ectopic lignification of the mesocarp (Gu et al., 1998; Muller et al., 2001; Smykal et al., 2007; Jaakola et al., 2010); this and other abnormalities could be responsible for premature rupture of the fruit wall. Thus, our data from Papaveraceae show that their *FUL-like* genes 1) function in proper floral meristem and sepal identity, similar to *AP1* and other *euAP1* genes, and 2) are required for proper fruit wall growth and cell differentiation, similar to *euFUL* genes.

We have further shown that down-regulation of both *FUL-like* copies in opium poppy results in additional phenotypes that were not observed in doubly down-regulated California poppy plants (Fig. 3.6). This may be a consequence of the occurrence of different gene duplication events in *Papaver* and *Eschscholzia* (N. Pabón-Mora and A. Litt unpublished data). Double *papsfl1-fl2* mutant plants exhibit delayed flowering, and a similar role in the reproductive transition has been reported for *FUL-like* and *euFUL* genes (Murai et al., 2003; Immink et al., 1999). However, down-regulation of California poppy *FUL-like* genes does not appear to affect flowering time.

Because this phenotype is not universal for down-regulated Papaveraceae *FUL-like* genes, and because all down-regulated opium poppy plants eventually did flower, it is likely that *FUL-like* genes are redundant with other key genes that control the transition to flowering, as has been suggested for *FUL* in Arabidopsis (Ferrandiz et al., 2000).

In addition, *papsfl1-fl2* double mutant plants exhibit mosaic outer petals with large green patches and abnormal epidermal cell identity (Fig. 3.6A–I). This suggests that in opium poppy, *FUL-like* genes, similar to Arabidopsis *AP1*, are required for proper specification of floral meristem and perianth identity. The loss of sepal identity is linked to the loss of floral identity; likewise, the loss of petal identity may be linked to loss of floral identity, or may be an independent function of *FUL-like* genes. No other *AP1/FUL* homolog besides *APETALA1* has been shown to be an A-function gene as defined in the ABC model of floral organ identity (Coen and Meyerowitz, 1991), specifying both sepal and petal identity (e.g., Berbel et al., 2001; Taylor et al., 2002; Murai et al., 2003; Benlloch et al., 2006; Melzer et al., 2008;). Nonetheless, two major differences can be noted in the function of opium poppy *FUL-like* genes and Arabidopsis *AP1* in the second whorl: 1) in opium poppy only the outermost whorl of petals is affected whereas in Arabidopsis all petals, which are in a single whorl, are affected, and 2) the second whorl organs of opium poppy consist of mosaic organs with both petal and leaf- or carpel-like epidermal cells, suggesting a homeotic transformation, whereas in Arabidopsis the second whorl petals are absent and instead the second whorl consists of ectopic meristems in the axils of the leaf-like first whorl organs (Irish and Sussex, 1990; Bowman et al., 1993). Therefore, although in both cases down-regulation of *AP1*/*FUL* lineage genes results in loss of sepal and petal identity, the phenotype in the second whorl is different enough to suggest differences in the developmental pathways leading to loss of petal identity.

In addition to specifying floral organ identity, A-function and C-function genes are also expected to repress each other, thereby defining the boundary between the outer sterile and inner reproductive organs of the flower (Coen and Meyerowitz, 1991; Bowman et al., 1993; Gustafson-Brown et al., 1994). In Arabidopsis, AP1 plays a role in regulating *AG* expression through formation of a complex with SEUSS and LEUNIG (Sridhar et al., 2004, 2006), however loss of *AP1* function does not result in ectopic expression of *AG* (Bowman et al., 1991; Gustafson-Brown et al., 1994; Yanofsky, 1995) or carpeloid characteristics in the first whorl (Irish and Sussex, 1990; Coen and Meyerowitz, 1991; Ferrandiz et al., 2000). In wild type opium poppy flowers, *AG* homologs are expressed at low levels in sepals and petals (Hands et al., 2011), thus C-class gene expression is not restricted to the inner two whorls as in Arabidopsis. We showed that in opium poppy plants in which both *FUL-like* paralogs were downregulated, potential carpel-like epidermal cells are observed in the transformed sectors of second whorl organs (Fig. 3.6D–L). In addition we also showed that in these green petal sectors, *PapsAG1 and PapsAG2* expression does not increase in comparison to wild type petals, suggesting that misregulation of *PapsAG* is not responsible for the change in epidermal identity. These data suggest that although the opium poppy *FUL-like* genes specify sepal and petal identity they do not appear to regulate C-function gene expression, one of the components of the A-function specified by the ABC model (Coen and Meyerowitz, 1991). Carpel-like cell identity may be the result of persistent wild type expression of C-class genes in the second whorl after loss of *FUL-like* gene function.

3.3.c. Opium poppy FUL-like proteins form heterodimers in a yeast system.

Identical phenotypes were observed when either of the two *PapsFUL-like* genes was down-regulated; this is in agreement with the fact that PapsFL1 and PapsFL2 interact strongly in a yeast system, and suggests that they might act in planta as a heterodimer that is critical for proper floral meristem identity, sepal identity, repression of axillary meristem growth, and fruit development (Fig. 3.4 and 3.5). In addition, new phenotypes emerged when both *PapsFL1* and *PapsFL2* were simultaneously down-regulated, suggesting that they redundantly regulate flowering time and petal identity (Fig. 3.6). The fact that the flowering time phenotype is only manifest when both copies are down-regulated suggests that the two paralogs may function interchangeably in forming complexes with other proteins important in this process, such as SVP/AGL24 and SOC1 orthologs, as has been suggested for FUL and AP1 (DeFolter et al., 2005; Gregis et al., 2006). Likewise, in petal development, the opium poppy FUL-like proteins could be interacting with PapsAP3 and PapsPI in a fashion similar to the higher order protein complexes that AP1 (and other euAP1 genes) forms and that are thought to control floral organ identity in Arabidopsis and snapdragon (Davies et al., 1996; Egea-Cortines et al., 1999; Honma and Goto, 2001; Theissen, 2001).

3.3.d. The core-eudicot duplication in the *AP1/FUL* gene lineage resulted in subfunctionalization in *euFUL* and *euAP1* genes.

The available information on the evolution of the *AP1/FUL* gene lineage suggests a scenario in which a major duplication event, accompanied by protein sequence divergence of one resulting paralagous clade, coincides with the diversification of a large group of flowering plants, the core eudicots. Because basal eudicot *FUL-like* genes represent the *AP1/FUL* gene-type prior to the core-eudicot gene duplication, the data presented here allow us to gain an understanding of functional evolution in the core-eudicot *euFUL* and *euAP1* genes. Our data show that *FUL-like* genes in both species in the Papaveraceae are important in axillary meristem dormancy and in proper floral meristem and sepal identity; in addition, *FUL-like* genes exert a role in fruit development by promoting normal development of the fruit wall during fruit maturation (Fig. 3.5V–AA; 3.7S–V). Finally, our data from opium poppy suggest that *FUL-like* genes may also regulate cauline leaf development and flowering time and may be important for proper petal identity (Fig. 3.4D–F, 3.6A–I). These functions have been differentially retained in *euAP1* and *euFUL* genes, with the former functioning in floral meristem and sepal identity and the latter in the reproductive transition, floral meristem identity, fruit development, and axillary meristem activation (Irish and Sussex, 1990; Bowman et al., 1993; Gu et al., 1998; Ferrandiz et al., 2000; Berbel et al., 2001; Blázquez et al., 2006) Melzer et al., 2008; Jaakola et al., 2010). Therefore, our data suggest that the functions reported for *euFUL* and *euAP1* genes in core eudicots are each part of the original functional repertoire of pre-duplication *FUL-like* genes, and that following the core-eudicot duplication, the *AP1/FUL* gene lineage underwent subfunctionalization (Fig. 3.8). Some functions were retained redundantly in both *euAP1* and *euFUL* genes, such as specification of floral meristem identity; however, most functions appear to have been parceled out to one or the other paralog.

Our data also suggest that that the acquisition of different and characteristic motifs at the C-terminus of euAP1 proteins is not correlated with the acquisition of novel core eudicot-specific functions. Evidence regarding the importance of the euAP1 motifs for proper protein function is inconsistent. Yalovsky et al. (2000) showed that an AP1 protein with a mutated farnesylation motif was unable to completely recapitulate the normal overexpression phenotype for AP1; this suggests that farnesylation is required for proper protein function. However, chimeric AP1 proteins carrying the C terminal domain of AGAMOUS, which lacks a farnesylation or a transcription activation domain, were able to produce a typical over-expression *AP1* phenotype in Arabidopsis (Krizek and Meyerowitz, 1996). In addition, PEAM4 (the pea euAP1 protein), which lacks a farnesylation motif (Berbel et al., 2001), and monocot *FUL-like* genes, can complement the Arabidopsis *ap1* mutant (Chen et al., 2008), suggesting that these proteins with different C-terminal motifs can provide the same functions as AP1 in an Arabidopsis background. Our data also suggest that the novel C-terminal motifs of euAP1 proteins, even though they have been shown to be functional (farnesylation and transcription activation), do not in fact result in novel roles for these proteins. Nonetheless, we know AP1 and FUL each have unique functional roles in flower development; these data raise the possibility that these roles may be determined by differences in regulation rather than sequence. Further studies that explore regulation of *AP1* and *FUL* in Arabidopsis and complementation of *ap1* and *ful* mutants with *FUL-like* genes could help address some of these remaining questions.

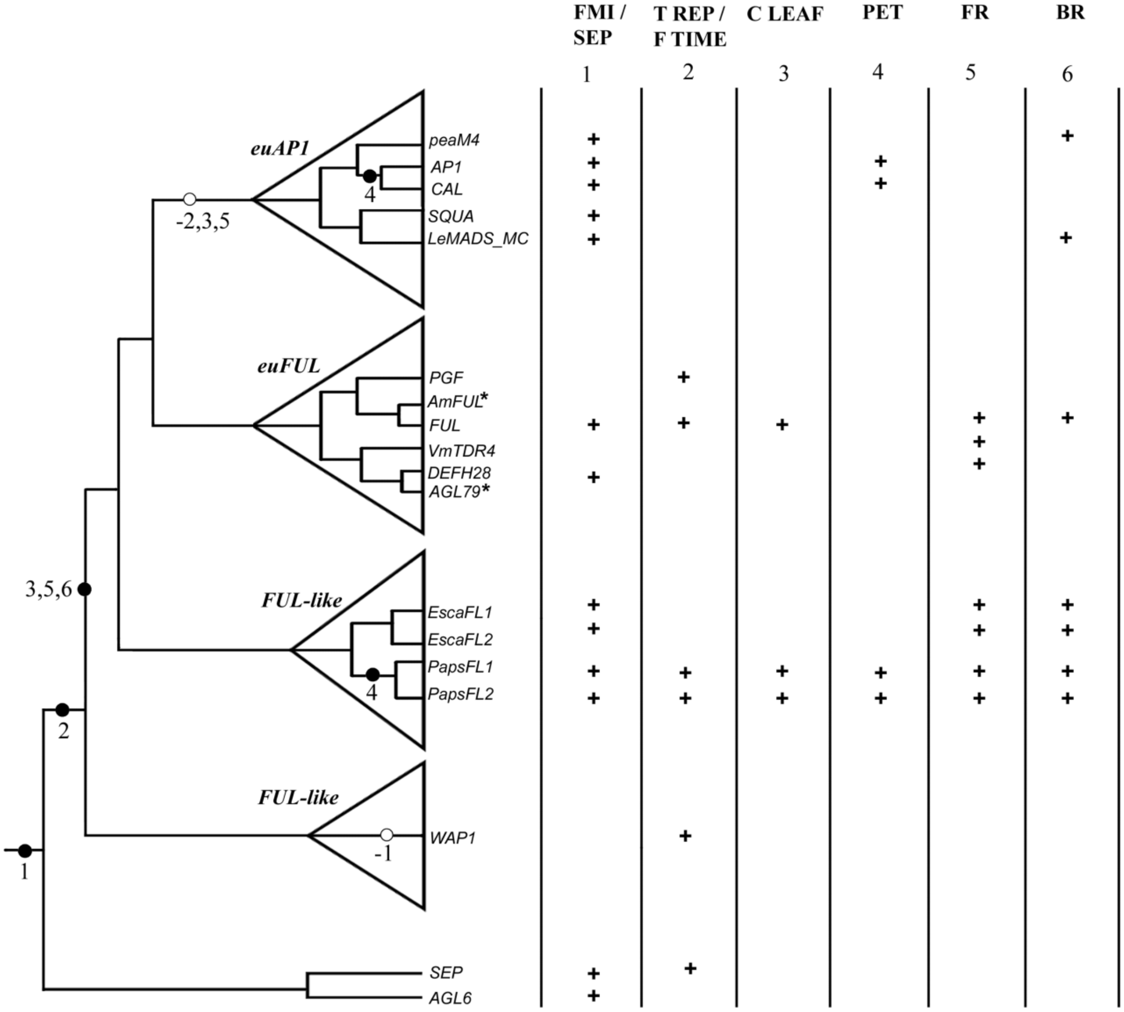


Figure 3.8. Optimization and mapping of functions recorded for *AP1/FUL* homologs. Based on the available data we hypothesize that the ancestral functions in the *AP1/FUL* gene lineage include floral meristem and sepal identity (1), because these are functions that are shared with the sister *SEPALLATA* and *AGL6* gene lineages. Transition to the reproductive meristem (2) appears to be ancestral just to the *AP1*/*FUL* lineage. Before the diversification of the Papaveraceae the genes acquired functions in cauline leaf development (3), branching (6), and fruit development (5), although the acquisition of these functions could have happened earlier than is shown in this figure. After the diversification of the core eudicots some of these functions (1, 6) were retained by both the *euFUL* and the *euAP1* clades, whereas others (2,3,5) were exclusively retained by members of the *euFUL* clade. A role in petal identity (4) appears to have been independently acquired in opium poppy and Arabidopsis. Asterisks = no functional data available. FMI/SEP, floral meristem identity and sepal identity; T REP/F TIME, transition to reproductive meristems/flowering time; C LEAF, cauline leaf development;; PET, petal identity; FR, fruit development; BR, branching. Closed black circles indicate gain of function (also numbered on the right), open white circles indicate loss of functions. + symbolizes that the function has been recorded for that gene.

In addition, a novel function, specification of petal identity, appears to have been acquired in parallel in different angiosperm lineages. To date this has only been observed in Arabidopsis (Irish and Sussex, 1990; Coen and Meyerowitz, 1991; Castillejo et al., 2005) and now in *Papaver*. The absence of a canonical A-function gene, controlling the identity of the two outer floral whorls, from any other core eudicot has raised questions regarding the universality of this element of the ABC model (Gutierrez-Cortines and Davies, 2000; Theissen et al., 2000; Shepard and Purugganan, 2002; Smyth, 2005; Litt, 2007; Causier et al., 2010; Litt and Kramer, 2010; Wollmann et al., 2010). It has been suggested that this role is unique to Brassicaceae, possibly due to Brassicaceae-specific duplications and functional diversification (Lowman and Purugganan, 1999). We demonstrate that *FUL-like* genes in opium poppy behave to a large degree as A-function genes, suggesting that *AP1/FUL* homologs have been independently co-opted in the determination of proper petal identity across different groups of flowering plants.

3.4. MATERIALS AND METHODS

3.4.a. Plant Material and Growth Conditions

Papaver somniferum cv. Persian white (opium poppy) seeds were obtained from J.L.Hudson, La Honda, California, USA. Seeds were germinated at 14 hours light/10 hours dark at 22ºC. Seedlings were grown in the same conditions. *Eschscholzia californica* (California poppy seeds were obtained from Seed Empire, Bellevue, Washington, USA. Seeds were germinated at constant light at 25ºC. Seedlings were grown in the same conditions.

3.4.b. Cloning of *FUL-like* genes

Partial sequences of opium poppy *FUL-like* genes were obtained from GenBank (accession numbers AY306177 and AY306178). These sequences were used to design primers for 3' Rapid Amplification of cDNA Ends (RACE). Total RNA was prepared from dissected organs, using TRIZOL reagent (Invitrogen) and was DNaseI (Roche) treated to remove residual genomic DNA. 5µg were used as template for cDNA synthesis with SuperScript III reverse transcriptase (Invitrogen). The resulting cDNA was diluted 1:10. 3' RACE was performed using FirstChoice® RLM-RACE Kit (Applied Biosystems) according to the manufacturer's instructions to get the complete nucleotide sequence at the end of the C-terminal domain and the 3'UTR of *PapsFL1* and *PapsFL2*. Degenerate primers (Litt and Irish 2003) were used to amplify California poppy (*Eschscholzia californica*) *FUL-like* genes. PCR products were cloned into pCR®2.1-TOPO® (Invitrogen) and sequenced.

3.4.c. RT-PCR

Expression of *FUL-like* genes was assayed in all floral organs at stages P7, P8, and anthesis, and in leaves and fruits. Total RNA was prepared from dissected organs as described above, from three different individuals. 3µg of RNA was used as template for cDNA synthesis (as described above). The resulting cDNA was diluted 1:10 and amplified using locus-specific primers (all primers listed in Supplemental Table 2). Reactions for *FUL-like* genes were run for 29 cycles at an annealing temperature of 55ºC. Reactions for *ACTIN* or UBIQUITIN (used as a loading control) were run for 31 cycles at an annealing temperature of 56 ºC and 47 ºC respectively. PCR products were run on a 1.2% agarose gel stained with ethidium bromide and digitally photographed using a DigiDoc-it® Darkroom UVP equipped with a Cannon PC1089 digital camera.

3.4.d. In Situ Hybridization and Anatomy

Developing shoot apical meristems in the vegetative and reproductive stages were collected from wild-type plants of opium poppy and California poppy and fixed under vacuum in freshly prepared FAA (50% ethanol, 3.7% formaldehyde and 5% glacial acetic acid). Samples were prepared and sectioned at 10µm according to standard methods (Langdale, 1993) on a Microm HM3555 rotary microtome. DNA templates for RNA probe synthesis were obtained by PCR amplification of 400- 550 bp fragments. To ensure specificity, the probe templates included 200-210 bp of the 3' untranslated region and approximately 300 bp of the coding region. Because *EscaFL1* and *EscaFL2* were more similar to each other than *PapsFL1* and *PapsFL2*, the DNA templates for California poppy included 500 bp of the variable K and C domain of *EscaFL1* and *EscaFL2* (Supplemental Fig. 2; Supplemental Table 2); nonetheless we cannot rule out the possibility that the probes could cross-hybridize. Digoxigenin-labeled RNA probes were prepared using T7 polymerase (Roche), Murine RNase Inhibitor (New England Biolabs) and RNA labeling mix (Roche) according to manufacturer's protocol. RNA in situ hybridization was performed according to Ferrandiz et al. (2000), optimized for each species: slides with opium poppy sections were hybridized overnight at 55ºC, whereas California poppy slides were hybridized overnight at 47ºC. Probe concentration was identical for all the experiments, including the antisense control hybridizations. In situ hybridized sections were subsequently dehydrated and permanently mounted in Permount® (Fisher).

For fruit anatomy, material was fixed, embedded, and sectioned as above but slides were directly stained with Johansen's safranin and 0.5% Astra Blue in 2% tartaric acid. All sections were digitally photographed using a Zeiss Axioplan microscope equipped with a Nikon DXM1200C digital camera.

Leaf clearings were made following Ellis et al. (2009). Macroscopic photographs of leaves and flowers were taken using a EOS Cannon Rebel XS digital camera or a Nikon DXM1200F digital camera adapted to a Nikon SMZ1500 stereoscope.

3.4.e. TRV-VIGS

TRV1 and TRV2 vectors were provided by V. Irish (Yale University) and E. Kramer (Harvard University). A 630 bp fragment of *PapsFL1* (Supplemental Fig. 2) was amplified including a portion of the K domain, the C domain and a portion of the 3'UTR from floral bud cDNA using primers that added *Eco*RI and *Xba*I restriction sites to the respective 5' and 3' ends of the PCR product (Supplemental Table 2). A 590 bp fragment of *PapsFL2* (Supplemental Fig. 2) was amplified including a portion of the K domain, the C domain and a portion of the 3'UTR adding *Bam*HI to the 3' end and using a naturally occurring *Xba*I site in the 5' end of the PCR fragment. The PCR products were cloned into the pCR®2.1-TOPO® plasmid vector (Invitrogen), then digested with *Eco*RI (Roche) and *Xba*I (Roche) for *PapsFL1,* and *Xba*I and *Bam*HI (Roche) for *PapsFL2*. Fragments were ligated into the similarly-digested TRV2 vector using T4 DNA Ligase (New England Biolabs) according to the manufacturer's protocol. This created two TRV2 constructs: TRV2-*PapsFL1* and TRV2-PapsFL2. A construct carrying fragments of both

PapsFL1 and *PapsFL2* fragments was also designed, but proved to be ineffective, therefore a mixture of both TRV2 vectors was used to silence both copies simultaneously (see below).

A similar strategy was used to create TRV2-*EscaFL1* and TRV2-*EscaFL2*. A 508 bp fragment of *EscaFL1* and a 500 bp fragment of *EscaFL2* (Supplemental Fig. 2) excluding the MADS domain and the first 15 amino acids of the K domain were amplified from floral bud cDNA using primers that added *Kpn*I and *Sac*I restriction sites to the 5' and 3' ends, respectively, of the PCR products. Cloning, digestion and ligation were as for TRV2-*PapsFL1* and TRV-*PapsFL2*. All vectors (TRV1, TRV2-*PapsFL1*, TRV2-*PapsFL2*, TRV2-*EscaFL1,* TRV2-*EscaFL2* and TRV2-empty) were sequenced and separately transformed into *Agrobacterium tumefaciens* strain EHA105.

Agrobacterium growth and plant infiltration methods followed Drea et al., 2007; Wege et al., 2007 and Orashakova et al., 2009 and were modified as follows. Agrobacterium was resuspended to OD_{600}= 2.0 in 5% sucrose. For infiltration TRV2-target gene was mixed independently with TRV1 in equal volumes. For opium poppy 100 seedlings were transformed with TRV1 and TRV2-*PapsFL1,* 80 seedlings were transformed with TRV1 and TRV2-*PapsFL2*, and 108 were transformed with TRV1 and TRV2-*PapsFL1* and TRV2-*PapsFL2.* For California poppy, 60 seedlings were transformed with TRV1 and TRV2-*EscaFL1* and 60 seedlings were transformed with TRV1 and TRV2-*EscaFL2*. 40 seedlings per species were transformed with TRV1 and TRV2-empty. 20 wild type seedlings per species were grown side by side with the transformed plants as a control. To inoculate, incisions were made in the hypocotyls of each seedling with a needle and a drop of the Agrobacterium suspension was placed on the wound.

Screening in opium poppy

The treatment resulted in 5-20% mortality of opium poppy plants in each group of transformants. 4-5 weeks after transformations, tissue from the youngest leaves, cauline leaves, sepals and fruits was collected from all opium poppy plants including those treated with the experimental construct or the empty TRV2 vector, and wild type. 85 poppy plants treated with TRV1+ TRV2-*PapsFL1,* 69 poppy plants treated with TRV1 + TRV2-*PapsFL2*, 20 plants treated with TRV1+ TRV2-E and 3 wild-type plants were evaluated for target gene expression. RNA extraction, cDNA synthesis, and RT-PCR, were performed as described above for RT-PCR.

The levels of *PapsFL1* and *PapsFL2* mRNA relative to *ACTIN* were determined in wild type and in VIGS-treated plants using semi-quantitative RT-PCR. *PapsFL1* and *PapsFL2* primers amplify a region outside the fragments used in the TRV constructs (Supplemental Table 2). Aliquots of PCR products were removed every two cycles from cycle 25 to 35 and were run on a gel to identify the linear range of amplification. These were determined to be 28-32 cycles for *ACTIN*, 28-30 cycles for *PapsFL1* and 30-32 cycles for *PapsFL2.* PCR reactions were conducted using 1:20 dilutions of template cDNA with 31 cycles of amplification for *ACTIN* and 29 cycles of amplification for *PapsFL1* and *PapsFL2.* The products were separated by electrophoresis on a 1% agarose gel containing 0.1 mg/L ethidium bromide. Relative amounts of *PapsFL1* and *PapsFL2* were compared in the treated plants that showed silencing phenotypes versus the

TRV2-empty vector treated plants and the wild type plants. Gels were photographed as described above. Reactions were repeated two additional times.

To screen for the presence of the vector, cDNA was synthesized from each RNA sample using a reverse primer in the vector sequence. The presence of both TRV1 and TRV2 in cDNA samples from VIGS-treated plants and control untreated plants was assessed by RT-PCR using vector-specific primers (Hileman et al., 2005).

Screening in California poppy

The treatment resulted in 3-11% mortality of California poppy plants in each group of transformants. To test for down-regulation and presence of the vector, tissue was collected as for opium poppy from 3 wild type plants and transformed plants that showed phenotypic changes: 30 plants treated with TRV2-*EscaFL1* and 17 plants with TRV2-*EscaFL2* (total n=47). RNA was prepared as described above for all the samples. The relative mRNA levels of *EscaFL1* and *EscaFL2* were determined in wild type and in VIGS-treated plants using semi-quantitative RT-PCR as described above for opium poppy (Supplemental Table 2). *ACTIN* levels were variable between different sample tissues collected for *E. californica*, so *UBIQUITIN* was used instead as a control. The linear range of amplification from these loci was 30-32 cycles for *UBIQUITIN*, and 28-30 for *EscaFL1* and *EscaFL2.* PCR products were prepared using 1:20 dilutions of template cDNA with 31 cycles of amplification for *UBIQUITIN* and 29 cycles of amplification for *EscaFL1* and *EscaFL2.* Gels were photographed as described above. Reactions were repeated two additional times.

3.4.f. qRT-PCR

To confirm down-regulation and to better evaluate the reduction of transcript in the down-regulated plants, a subset of samples that by semi-quantitative RT-PCR showed mild, moderate and strong down-regulation was subjected to quantitative RT-PCR. qRT-PCR reactions were carried out using the 7300 qPCR system and SDS software (Applied Biosystems). Primers used for quantification of transcripts were designed using Primer Express ™ v 3.0 (Applied Biosystems) (Supplemental Table 2). qRT-PCR was performed in total volumes of 25 µl containing 12.5 µl FastStart Universal SYBR® Green (Roche), 4µl diluted cDNA, 2.5 µl each PCR primer (1µM) and 3.5µl deionized water. PCR conditions were 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of denaturation at 95°C for 15 sec and annealing-extension at 60°C for 2 min. Three genes, *Elf1a*, *ACTIN*, and *GADPH* were tested as endogenous controls; *GAPDH* showed consistency and expression in the appropriate range across different tissues and therefore was used as the constitutive reference transcript (Yellina et al., 2010). The level of *FUL-like* mRNA in down-regulated leaf tissue was analyzed relative to wild type using the 2-ΔΔCt method (Livan and Schmittgen, 2001). Standard error is reported for three technical replicates of each sample.

3.4.g. Yeast Two Hybrid Analysis

Full length *PapsFL1* and *PapsFL2* were amplified from floral bud cDNA using primers that added *Nde*I and *Eco*RI restriction sites to the respective 5' and 3' ends of the PCR product (Supplemental Table 2). Each full coding sequence was fused with the GAL4 binding domain in the pGBKT7 vector (Clontech) and the GAL4 activation domain in the pGADT7 vector

(Clontech). Single constructs were transformed into yeast strain AH109. All constructs show minimal autoactivation upon the addition of 2.5mM 3AT. To test interactions, pairwise combinations of constructs were transformed into AH109. Homo- and heterodimerization were tested by growing colonies on selective medium (SD). Growth was measured after 3 days and after 6 days. Interactions between each vector and an empty vector were used as negative controls.

3.4.h. Scanning Electron Microscopy (SEM)

For scanning electron microcopy studies, leaves, cauline leaves, floral buds, dissected floral organs and fruits from wild-type and down-regulated plants of opium poppy and California poppy were fixed under vacuum in FAA and stored in 70% ethanol. For analysis, material was dehydrated through an ethanol series and critical point dried using a Samdri 790 CPD. Material was mounted on aluminum stubs with adhesive tabs (Electron Microscopy Sciences), sputter coated with gold palladium in a Hummer 6.2 sputter coater; and examined and photographed at 10kV in a Jeol JSM-5410 LV Scanning Electron Microscope.

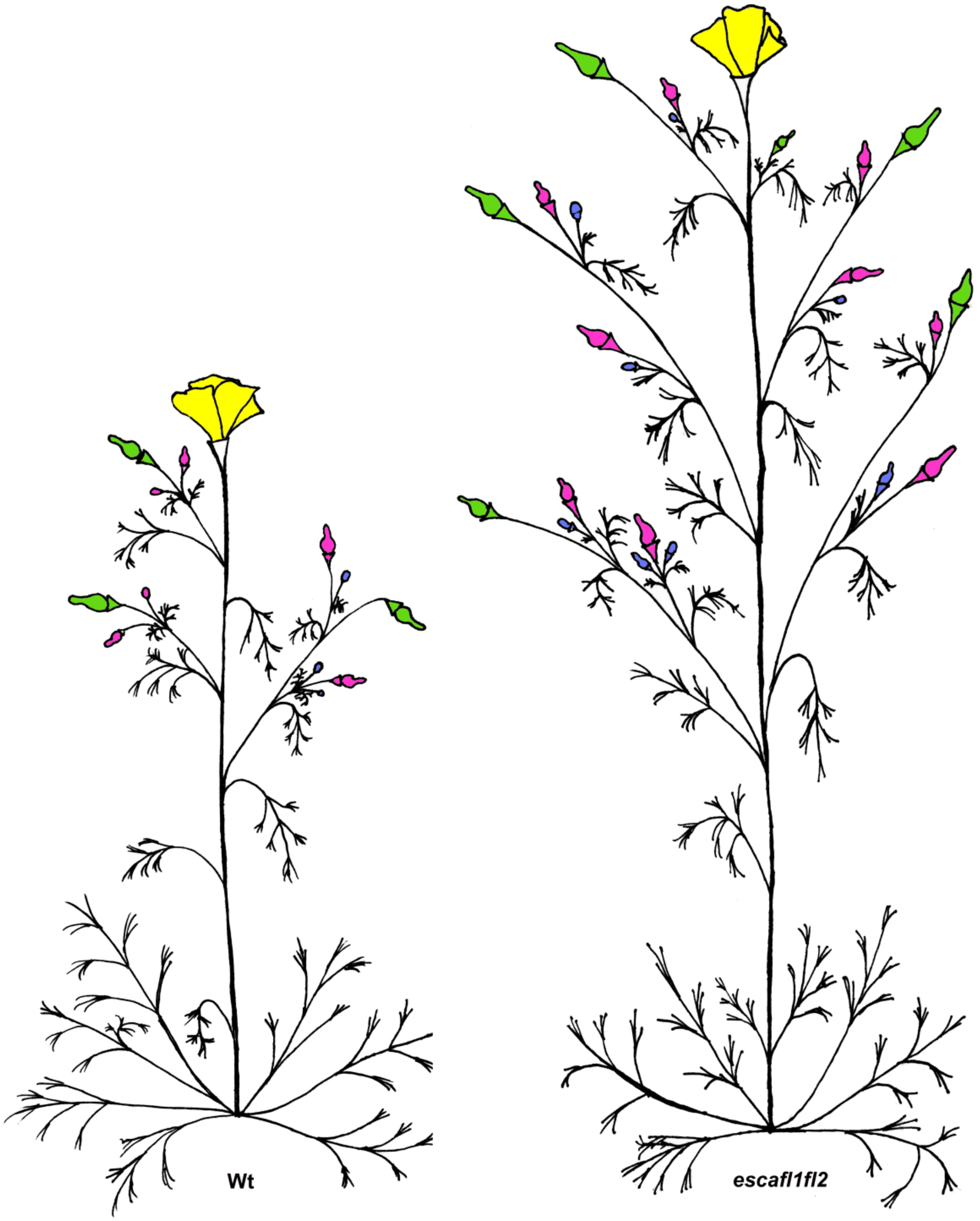
3.4.i. Accession numbers

Sequence data can be found in the EMBL/GenBank data libraries under accession numbers AY306177, AY306178, HM592297, and HM592298.

3.5. APPENDIX

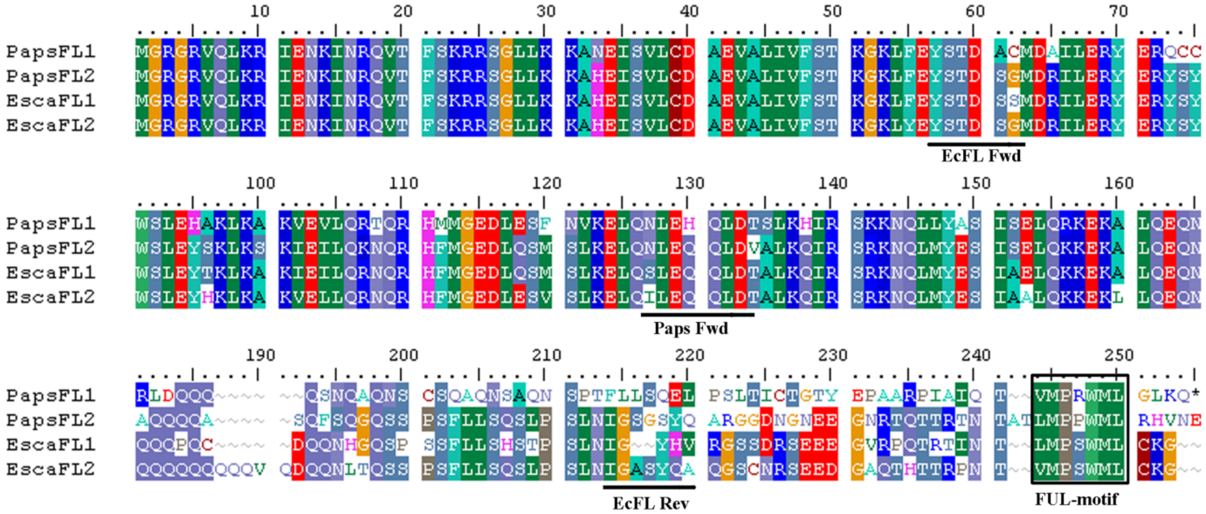
3.5.a. Supplemental Figure 1. Diagram showing differences in first, second, and third order branching in California poppy wild type and *escafl1fl2* plants.

Wild type (left) and escafl1fl2 plants (right). The terminal flower is yellow, first order flowers are green, second order branches are pink, third order branches are purple.



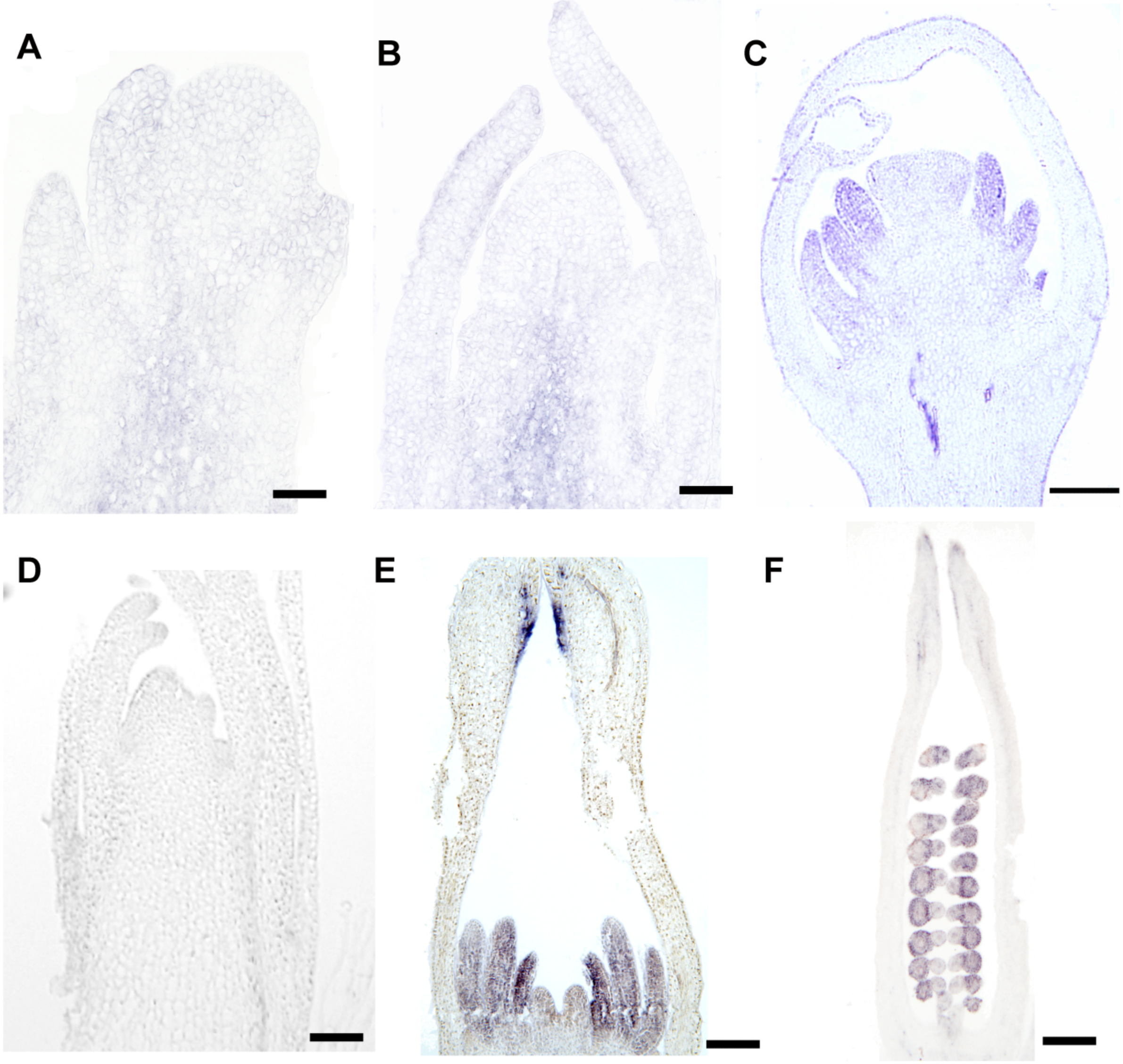
3.5.b. Supplemental Figure 2. Amino acid alignment of the opium poppy and California poppy FUL-like proteins.

FUL-like motif is indicated by the box. Lines below indicate the locations of the primers used to amplify the regions for VIGS constructs from the *PapsFUL-like* genes from opium poppy and the *EscaFUL-like* genes from California poppy. *PapsFL1-PapsFL2* reverse primers are not shown because they bind to the 3'UTR. EcFL Fwd, forward *EscaFL* primer; EcFL Rev, reverse *EscaFL* primer; PapsFL Fwd, forward *PapsFL1*- *PapsFL2* primers.



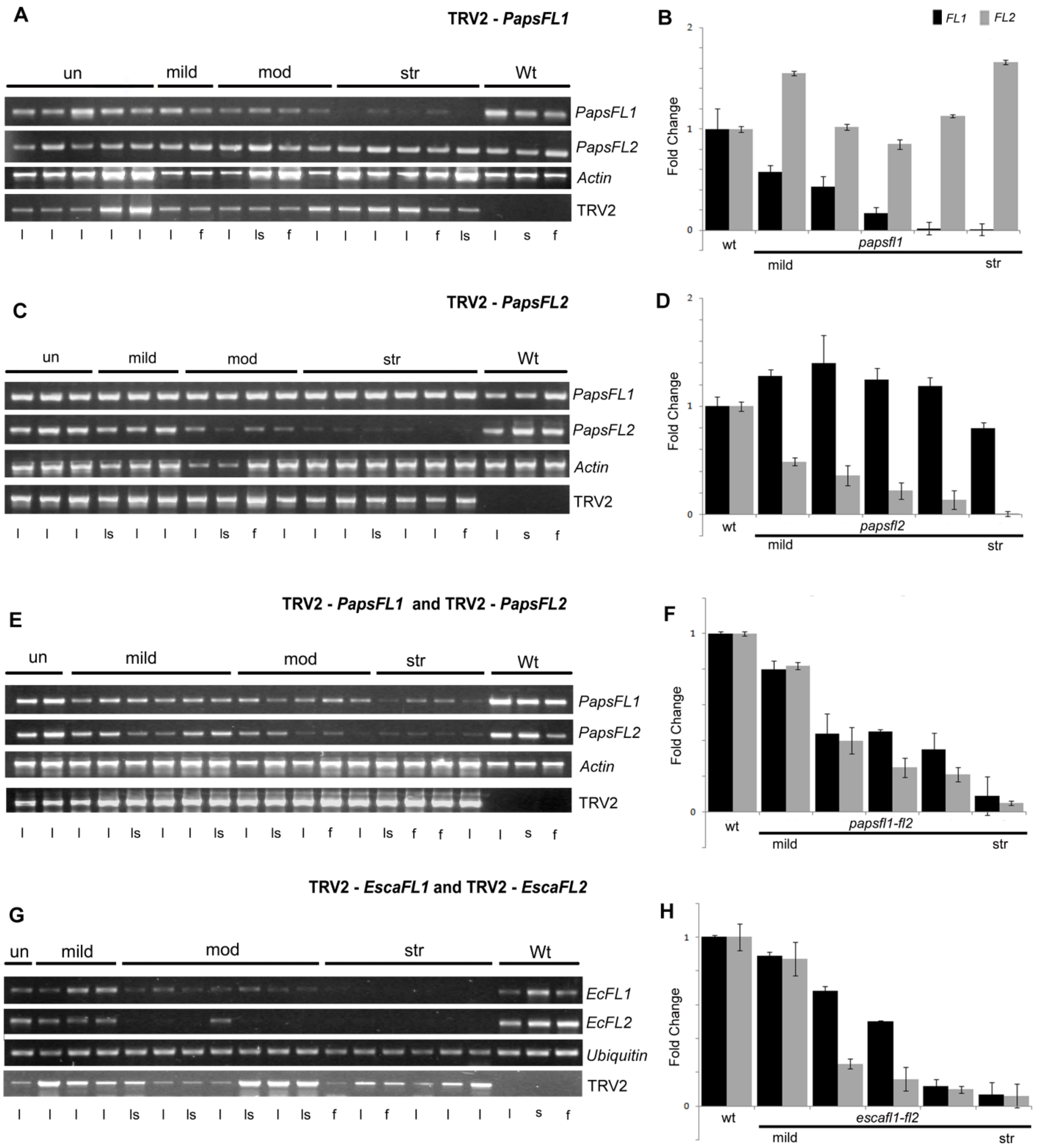
3.5.c. Supplemental Figure 3. In situ hybridization controls in *Papaver somniferum* and *Eschscholzia californica*.

(A) to **(C)** Controls for opium poppy. **(A)** *PapsFL1* sense probe. **(B)** *PapsFL2* sense probe. **(C)** Expression of *PapsPI* used to test the specificity of the technique in light of the broad expression of *PapsFL1*; results are similar to those obtained by Drea et al. (2007)
(D) to **(F)** Controls for California poppy **(D)** *EscaFL* sense probe. **(E)** and **(F)** Expression of *EcAG* used to test the specificity of the technique in light of the broad expression of *EscaFUL-like* genes; results are similar to those of Zahn et al. (2006). Scale bars: 50 µm



3.5.d. Supplemental Figure 4. Locus-specific RT-PCR and qRT-PCR using cDNA prepared from organs of VIGS-treated plants.

(A), (C) and (E) RT-PCR analysis of opium poppy plants transformed with **(A)** TRV2-*PapsFL1,* **(C)** TRV2-*PapsFL2* and **(E)** TRV2-*PapsFL1* and TRV2-*PapsFL2* simultaneously, showing preliminary categories of un-silenced (un), mildly (mild), moderately (mod), and strongly (str) silenced plants. Samples were extracted from leaves (l), leaf-like sepals (ls) or fruits (f) and screened for down-regulation of *PapsFL1* and *PapsFl2* as well as presence of the vector (TRV2). Note specific down-regulation of *PapsFL1* in **(A)***,* specific down-regulation of *PapsFL2* in **(C)** and down-regulation of both copies in **(E)**. Samples of wild type leaf (l), sepal (s) and fruit (f) were used for comparison and *ACTIN* was used as a control.
(B), **(D)** and **(F)** qRT-PCR of a subset of leaf samples showing the range of silencing (from mildly to strongly down-regulated) of **(B)** *PapsFL1*, **(D)** *PapsFL2* and **(F)** *PapsFL1* and *PapsFL2* simultaneously. Fold change in *PapsFL1* (black bars) and *PapsFL2* (gray bars) expression in samples is shown relative to the wild type leaf gene expression. Error bars show ± SD among three technical replicates. *GADPH* was used as the endogenous control.
(G) RT-PCR analysis showing preliminary categories of un-silenced (un), mildly (mild), moderately (mod) and strongly (str) silenced tissue samples from California poppy plants transformed with TRV2-*EscaFL1* and TRV2-*EscaFL2*. Samples were collected from the same organs as opium poppy plants. Treated plants were screened for down-regulation of *EscaFL1* and *EscaFl2* and for presence of the vector (TRV2). Note down-regulation of both *EscaFL1* and *EscaFL2*. Similar wild type samples were used for comparison and *UBIQUITIN* was used as a control.
(H) qRT-PCR of a subset of leaf samples showing the range of silencing (from mildly to strongly down-regulated) of *EscaFL1* and *EscaFL2* simultaneously. Fold change in *EscaFL1* (black bars) and *EscaFL2* (gray bars) expression is shown relative to the wild type leaf gene expression. Error bars show ± SD among three technical replicates. *GADPH* was used as the endogenous control.


A
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str
Wt
PapsFL1
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PapsFL2
Actin
TRV2
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TRV2 - EscaFL1 and TRV2 - EscaFL2
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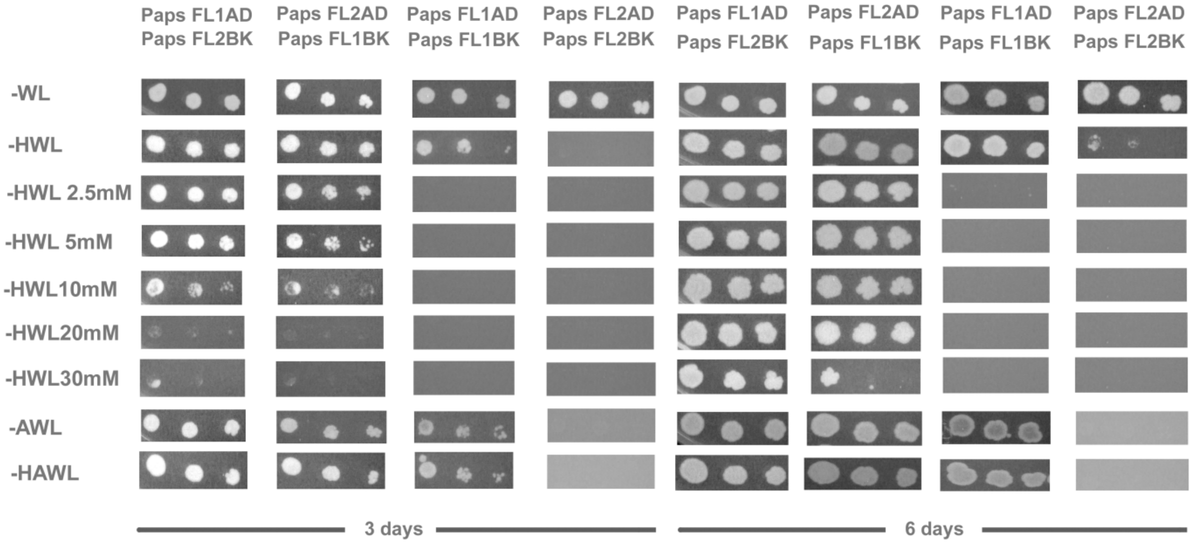
3.5.e. Supplemental Figure 5. Protein interactions between PapsFL1 and PapsFL2 as determined by growth on selective SD medium.

Opium poppy proteins were cloned into both activation domain (AD) and binding domain (BK) vectors. Within each column corresponding to a interacting pair, the three columns of colonies represent a dilution series (10^{-5}, 10^{-4} and 10^{-3} colony forming units) of each strain grown on SD medium (-HWL, -AWL and –HAWL). The –HWL medium was supplemented with 2.5 to 30mM 3-amino- 1,2,4 –triazole.



3.5.f. Supplemental Figure 6. Range of variation of the leaf-like sepal phenotype in *escafl1-fl2* California poppy plants.

(A) to **(C)** Slightly abnormal sepals, with leafy edges. In these flowers, floral organs remain trapped inside because sepals are largely fused but not deciduous. Floral cup is visible.
(D) to **(I)** Strongly abnormal, free (un-fused) sepals with highly dissected leaf-like edges that allow full or partial exposure of the remaining floral organs. Floral cup is present.
(J) to **(L)**. Complete homeotic transformation of sepals into leaf- like organs, where no remnants of the floral cup are observed. Scale Bars: 0.5 cm

3.5.g. Supplemental Table 1. Summary of phenotypes identified using VIGS to silence poppy *FUL-like* genes individually and simultaneously

Construct /(effectiveness[a])	Phenotypes	Nºplants
TRV2-PapsFL1/ (27/100)	Branched inflorescences	21
	Overgrowth and shape defects in cauline leaves	6
	Leaf –like sepals	8
	Carpel defects /Premature fruit rupture	4
TRV2-PapsFL2/ (13/80)	Branched inflorescences	10
	Overgrowth and shape defects in cauline leaves	5
	Leaf –like sepals	7
	Carpel defects /Premature fruit rupture	3
TRV2-PapsFL1 and TRV2-PapsFL2/ (15/108)	Delay in reproductive transition	12
	Branched inflorescences	7
	Overgrowth and shape defects in cauline leaves	5
	Leaf –like sepals	5
	Mosaic green petals	5
	Carpel defects /Premature fruit rupture	5
TRV2-EscaFL[b] (40/120)	Branched inflorescences	40
	Leaf –like sepals	40
	Carpel defects /Premature fruit rupture	4

[a] effectiveness: (Total number of plants down-regulated/ Total number of plants transformed)

[b] both constructs downregulated both gene copies therefore data are combined here.

3.5.h. Supplemental Table 2. Primers used for all the experiments in chapter 3

	Primer name	Primer sequence
In Situ Hybridization	*PapsFL1*- F	GCACCAACTTGATACTTCC
	PapsFL1- R-T7	CTTAATACGACTCACTATAGGGTGATCCCTGATTGCTTTCTT
	PapsFL2- F	AAGAGCTCCAAAATCTAG
	PapsFL2- R-T7	CTTAATACGACTCACTATAGGGAGATACAACATTACATGCA
	PapsPI F	AGTGGAAATTCATGGAAGAGG
	PapsPI R T7	CTTAATACGACTCACTATAGGGAGTTATAGTAGCAGCTATGATC
	EcFL1- F	GGAAAACTCTTTGAATACTCTACTGATTCCAGCA
	EcFL1- R-T7	CTTAATACGACTCACTATAGGGTCGCTGCTGCCTCGAACATGGTA
	EcFL2- F	GCTCTATGAATACTCCACTGATTCTGGTA
	EcFL2- R-T7	CTTAATACGACTCACTATAGGGTCCTTGCGCCTGATAACTTG
	EcAG F	GCAACCAAACTGCGTCAACAAATCG
	EcAG R T7	CTTAATACGACTCACTATAGGGTGTCTGCTCCTGGTGGGAGTAATG
Yeast 2 Hybrid	*PapsFL1*- *Nde*I	CATATGGGAAGAGGTAGGGTTCAGCTGAAG
	PapsFL1- *Eco*RI	GAATTCCTATTGTTTGAGACCAAGCATCCA
	PapsFL2- *Nde*I	CATATGGGAAGAGGTAGGGTTCAGCTGAA
	PapsFL2- *Eco*RI	GAATTCTTCATTAACATGGCGAAGCATCCA
VIGS Insert	*EcFL1*- *Kpn*I F	GGTACCGGAAAACTCTTTGAATACTCTACTGATTCCAGCA
	EcFL1- *Sac*I R	GAGCTCTCGCTGCTGCCTCGAACATGGTAA
	EcFL2- *Kpn*I F	GGTACCGCTCTATGAATACTCCACTGATTCTGGTA
	EcFL2- *Sac*I R	GAGCTCCCTTGCGCCTGATAACTTGCACCAATG
	PapsFL1-*Eco*RI F	GAATTCTTGGAGCACCAACTTGATACTTCCT
	PapsFL1-*Xba*I R	TCTAGAAAGCACTGACCGTGTTCATG
	PapsFL2- F	AAGAGCTCCAAAATCTAGA
	PapsFL2-*Bam*HI R	GGATCCGAATAGAGATACAACATTACATGCATG
Locus specific RT-PCR	TRV2 - pYL156 F	GGTCAAGGTACGTAGTAGAG
	TRV2 - pYL156R	CGAGAATGTCAATCTCGTAGG
	ACTIN F	GATGGATCCTCCAATCCAGACACTGTA
	ACTIN R	GTATTGTGTTGGACTCTGGTGATGGTGT
	PapsFL1 F	CAATAGAAAGATACTTACTTCCATGGT
	PapsFL1 R	AAGCACTGACCGTGTTCATG
	PapsFL2 F	GTAACCGAATAATCTCATATCTATCTC
	PapsFL2 R	GAATAGAGATACAACATTACATGCATG
	UBIQUITIN F	AACCCTTGAGGTTGAATCATCC
	UBIQUITIN R	GTCCTTCTTTCTGGTAAACGT
	EcFL1- F	GGAAAACTCTTTGAATACTCTACTGATTCCAGCA
	EcFL1- R	TCGCTGCTGCCTCGAACATGGTAA
	EcFL2- F	GCTCTATGAATACTCCACTGATTCTGGTA
	EcFL2-R	TCCTTGCGCCTGATAACTTG
VIGS Down-regulation screen	*PapsFL1* F	GATTTAGAATCCTTCAATGTGA
	PapsFL1 R	AAGCACTGACCGTGTTCATG
	PapsFL2 F	GACTTACAATCCATGAGTCTTAAA
	PapsFL2 R	GAATAGAGATACAACATTACATGCATG
	EcFL1- F	TCTCTGTTCTTTGTGATGCTGAAGT
	EcFL1- R	TTGATGGTTCTAGTCTGTGGTCGAA
	EcFL2- F	GAAGTTGCTTTAATTGTCTTCTCTA
	EcFL2-R	AGGCCTCGTCGTATGTGTTTGTGC
qRT-PCR	*PapsFL1* F	TTATGCCACGGTGGATGCT
	PapsFL1 R	ACAGATCTATATGAAACATCCATCAACA
	PapsFL2 F	GAAGAAGGGAATCGAACTCAGACT

	PapsFL2 R	TGCGACGGATAGACACCCA
	EcFL1- F	ACAGCAGCAACCTCAGTGTGA
	EcFL1- R	TGCTGCCTCGAACATGGTAA
	EcFL2- F	ATAACCCAGCAGCAGCAGCAGCA
	EcFL2-R	GTATGTGTTTGTGCTCCATCTGTGTT
	GADPH-F	GCTTCCTTCAACATCATTCC
	GADPH-R	AGTTGCCTTCTTCTCAAGTC

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CHAPTER FOUR

The *Aquilegia FRUITFULL-like* gene has been co-opted into the leaf morphogenesis developmental network

4.1 INTRODUCTION

The *APETALA1/ FRUITFULL* (*AP1*/*FUL*) MADS-box transcription factors have been implicated in multiple developmental processes, from phase transition to floral organ identity to fruit development; however, how these functions were acquired over evolutionary time is unclear. It has been shown that the *AP1/FUL* genes are angiosperm specific and have undergone several duplication events. The most significant one correlates with the origin of the core eudicots, resulting in the *euAP1* and the *euFUL* clades (Litt and Irish, 2003; Shan et al., 2007). Sequence analyses have shown that proteins in each clade have divergent motifs at the C-terminus (Cho et al., 1999; Yalovsky et al., 2000; Litt and Irish, 2003; Shan et al., 2007); whereas functional studies demonstrate that they have some overlapping and some unique roles. Together, *euAP1* and *euFUL* genes are implicated in proper floral meristem identity and axillary meristem repression (Ferrándiz et al., 2000). Independently, *euAP1* genes play a role in sepal (and occasionally petal) identity (Irish and Sussex, 1990; Huijser et al., 1992; Berbel et al., 2001; Vrebalov et al., 2002; Benlloch et al., 2006), whereas *euFUL* genes control the transition to the reproductive meristem and cauline leaf growth, and play a role in fruit development (Gu et al., 1998; Immink et al., 1999; Muller et al., 2001; Melzer et al., 2008; Jaakola et al., 2010; Torti et al., 2012).

One way to assess the effect of gene duplication on the functional evolution of a gene lineage is to evaluate the plesiomorphic role of the lineage before the duplication event and to compare that role with the functions of the gene clades that resulted from the duplication. *FUL-like* genes, present in all flowering plants outside the core eudicots, represent the pre-duplication ancestors in the *AP1*/*FUL* gene lineage (Litt and Irish, 2003; Preston and Kellogg, 2006; Shan et al., 2007). In terms of sequence, FUL-like proteins are more similar to euFUL than to euAP1 proteins, but only scattered data are available regarding their function. Grass *FUL-like* genes have been implicated in the control of the transition from vegetative to reproductive meristems, based on the fact that transcripts of *FUL-like* genes accumulate during cold exposure in temperate grasses, and that wheat *apetala1-like* mutants (*wap1*) are unable to flower (Murai et al., 2003; Trevaskis et al., 2003; Trevaskis et al., 2007). On the other hand, *FUL-like* genes in poppies (basal eudicots) play pleiotropic roles in flowering time, cauline leaf development, proper floral meristem and perianth identity, as well as late roles in fruit development (Pabón-Mora et al., 2012). The poppy data suggest that several of the functions attributed to core eudicot paralogs were present before the duplication and were subsequently divided between *euAP1* and *euFUL* genes. However, the available functional information is very limited, confined to wheat (one of three paralogs) and two poppy species, and prevents us from generalizing on the role of *FUL-like* genes at this point.

MADS-box proteins are known to function as dimers or in higher order complexes (Theissen and Saedler, 2001; Smaczniak et al., 2011), so changes in their expression and interaction partners can also explain functional divergence in different homologs. *Arabidopsis AP1/FUL* genes can help to illustrate this. For instance, whereas *AP1* is expressed in the floral meristem and later on confined to sepals and petals (Bowman et al., 1993; Blázquez et al., 2006), *FUL* is expressed in the inflorescence meristem, cauline leaves and carpels (Mandel and Yanofsky, 1995; Gu et al., 1998; Ferrándiz et al., 2000). Both AP1 and FUL can interact in yeast with a common set of other MADS-box transcription factors involved in flowering transition, like SUPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1), inflorescence meristem identity, such as AGAMOUS-LIKE24 (AGL24), and floral organ identity, including SEPALLATA (SEP) 1/2/3/4, AGAMOUS-Like6 (AGL6) and AGAMOUS (AG) (Pelaz et al., 2001; DeFolter et al., 2005; Gregis et al., 2006; Kaufmann et al., 2010); however, AP1 has some exclusive floral organ identity partners including PISTILLATA (PI) and APETALA3 (AP3) (Riechmann et al., 1996; Honma and Goto, 2001; DeFolter et al., 2005). Thus, in *Arabidopsis* both changes in expression and protein partners could be responsible for the functional differences among paralogs. Outside of Arabidopsis, *euAP1* genes are expressed similarly to *AP1* (Hardenack et al., 1994; Ferrándiz et al., 2000; Berbel et al., 2001; Benlloch et al., 2006), whereas *euFUL* and *FUL-like* genes are more broadly expressed in vegetative parts, floral organs, and fruits (Gu et al., 1998; Fornara et al., 2004; Preston and Kellogg, 2007; Danilevskaya et al., 2008; Sather and Golenberg, 2009); Pabón-Mora et al., 2012). Protein interaction data from snapdragon (Huijser et al., 1992) and tomato (Leseberg et al.2008), have shown that other euAP1 proteins have similar interaction capabilities as AP1, whereas, the interactions of euFUL proteins in petunia, tomato and chrysanthemum and FUL-like proteins in rice and *Lolium* vary greatly, and occasionally have expanded to include typical euAP1 partners (Huijser et al., 1992; Immink et al., 2003; Fornara et al., 2004; Ciannamea et al., 2006; Leseberg et al., 2008; Immink et al., 2010). For the most part, the data hint to a more conserved euAP1 functional program in floral meristem determination and sepal identity and a more flexible recruitment of euFUL and FUL-like proteins into multiple processes. However, it also points to the need for more comparative functional data for *AP1/FUL* homologs, particularly outside of the core eudicots.

In order to evaluate the expression patterns, function and protein partners of *FUL-like* genes, we chose to work with the genus *Aquilegia* (Ranunculaceae). Its phylogenetic placement as a derived member within the order Ranunculaceae allows for a closer comparison with previously generated data from Papaveraceae species. Furthermore, important resources are available for *Aquilegia* including a complete genome sequence (http://www.phytozome.net) and an extensive EST database (Kramer and Hodges, 2010) . In addition, down-regulation of endogenous target genes using virus induced gene silencing (VIGS) has proven effective in several species of *Aquilegia* (Gould and Kramer, 2007; Kramer and Hodges, 2010). The present study explores the expression and function of the single *Aquilegia AP1/FUL* homolog, and shows that *AquilegiaFUL-like1* (*AqFL1*) shares some functions with its poppy counterparts, but has been co-opted to play a novel role in leaf morphogenesis. In contrast to all other *AP1/FUL* homologs characterized to date, *AqFL1* does not seem to function (or functions redundantly with other transcription factors) in flower or fruit development. We discuss these functional findings regarding *AqFL1* in the light of the known roles of *AP1/FUL* genes in core and non-core eudicots. In addition we highlight their implications for our current understanding of leaf development gene networks in angiosperms.

4.2. RESULTS

All species of *Aquilegia* are perennial herbs with leaves in a rosette, and most require a period of exposure to cold temperature (vernalization) prior to flowering (Kramer, 2009). Wild type *Aquilegia* leaves consist of three parts: 1) a leaf base with a large pair of stipules that enclose the younger leaves and the shoot apical meristem (SAM), 2) a petiole, and 3) a peltately-palmate compound lamina with three leaflets that are deeply segmented into secondary leaflets and have lobed margins. *Aquilegia* inflorescences are cymes (Tucker and Hodges, 2005). The inflorescence meristem forms two lateral bracts with axillary meristems before ending in a terminal flower. Lateral meristems retain the inflorescence meristem identity and repeat the same developmental pattern. Cauline leaves have short petioles and petiolules and are consistently smaller than rosette leaves. They vary in size and shape from the base to the apex of the inflorescence, often ranging from highly dissected lower leaves to leaves with three to one leaflet, with entire margins, subtending the lateral flowers. *Aquilegia* flowers are polysymmetric, with five free petaloid sepals, five free spurred petals, numerous stamens in 4-6 whorls and five staminodes that surround the five free carpels in the center of the flower (Tucker S.C., 2005; Ronse deCraene L., 2010). After fertilization, carpels become follicles that release the seeds through apical pores and sometime longitudinal slits.

4.2.a. *AqFL1* is the single member of the *AP1/FUL* gene lineage in *Aquilegia.*

Aquilegia possesses a single *FUL-like* gene in its genome (http://www.phytozome.net), unlike other Ranunculaceae which have two to four copies (Litt and Irish, 2003). *Aquilegia FUL-like 1* (*AqFL1*) falls within the Ranunculales *FUL-like1* gene clade, one of the two clades resulting from a Ranunculales-specific duplication (Fig. 4.1). *AqFL1* has a predicted Asparagine at amino acid position 123, a synapomorphy for all the Ranunculaceae sequences within the *FUL-like1* clade (Pabón-Mora *et al.* unpublished data). Sequence analysis predicts that *AqFL1* has the typical FUL-like C-terminal amino-acid motif (Fig. 4.1).

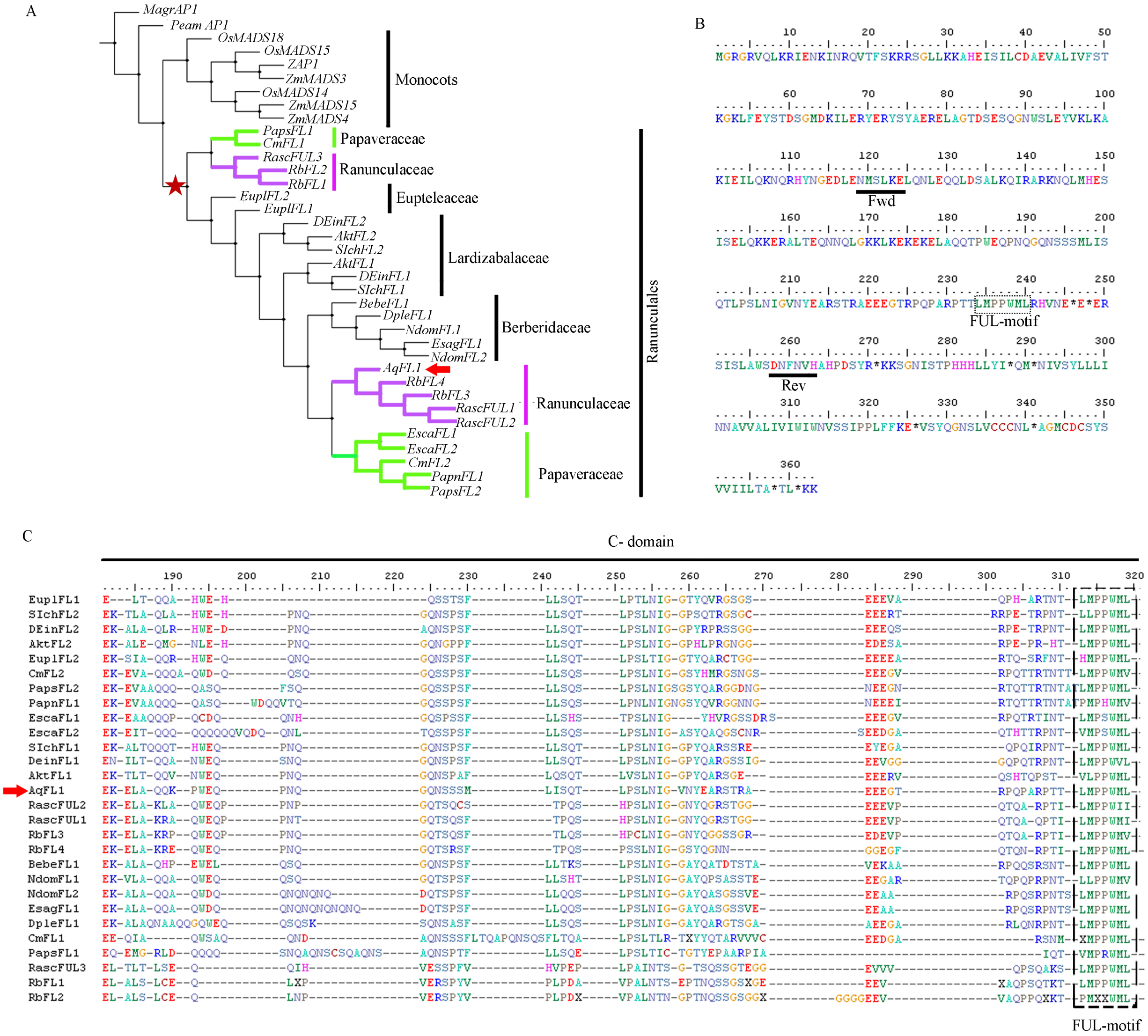


Figure 4.1. AqFL1 protein sequence and phylogenetic position. **(A)** The position of *AqFL1* among Ranunculales *FUL-like* genes (red arrow). Genes have been labeled with the family they were derived from. The green and purple clades are Papaveraceae and Ranunculaceae sequences, respectively. The topology shows a *FUL_like* duplication (star) event early during the diversification of the Ranunculales. **(B)** Amino acid sequence of AqFL1. FUL-motif is boxed. Positions of the primers used to amplify the region for the VIGS construct are underlined. **(C)** Alignment of several Ranunculaceae FUL-like proteins showing the variable C-terminal domain.

4.2.b. *AqFL1* is broadly expressed during vegetative and reproductive growth in *Aquilegia*.

We evaluated *FUL-like* gene expression in a series of dissected organs from *Aquilegia coerulea* using qRT-PCR (Fig. 4.2A). Results show that *AqFL1* is not detectable in cotyledons. Transcript is detected in very young shoot apical meristems and unfolding leaves before and after vernalization, but expression is stronger after vernalization. In addition *AqFL1* is expressed in all floral organs before anthesis, and in the developing fruit (Fig. 4.2A).

A more detailed examination of expression based on in situ mRNA hybridization shows that *AqFL1* is expressed in the shoot apex before vernalization as well as in the vasculature and the growing tips of young rosette leaves (Figs. 4.2B-D). As the leaves enlarge, *AqFL1* expression becomes restricted first to the lamina (Figs. 4.2B-D) and later to the growing tips (Fig. 4.2E). After the reproductive transition, *AqFL1* is expressed in young floral buds during sepal initiation (Figs. 4.2F-G), and in petal and stamen primordia as they differentiate (Figs. 4.2H-J). When the carpel primordia initiate, *AqFL1* expression is reduced in sepals, but remains in the tips of the elongating petals, stamen, staminodia and carpel primordia (Fig. 4.2K). These results show that *AqFL1* is expressed broadly in time and space during vegetative and reproductive growth as was shown previously by Ballerini and Kramer (2011).

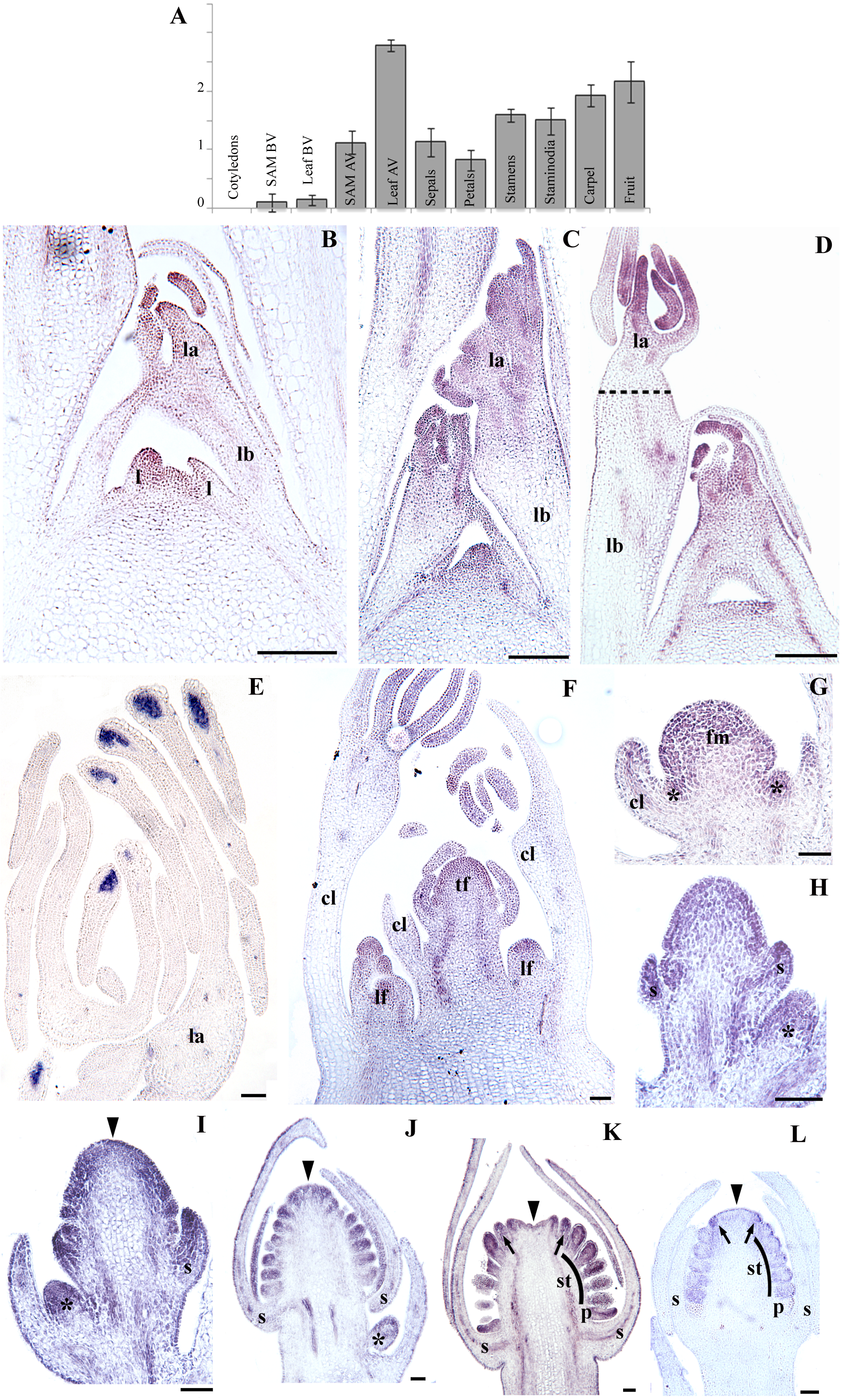


Figure 4.2. Expression of *AqFL1* in developing *Aquilegia*

(A) qRT-PCR results showing expression of *AqFL1* in leaf and shoot apical meristem (SAM) before and after vernalization, in dissected floral organs prior to anthesis, as well as in fruits and leaves. **(B)** to **(K)** In situ mRNA hybridization of *AqFL1.* **(B)** to **(D)** Shoot apical meristem before the transition to flowering showing very young leaf primordia in B, and older leaves in C and D. **(E)** Close up of a dissected leaf lamina of a rosette leaf; this section shows the vernation of the leaf in which segments correspond to overlapping growing tips of the leaflets. **(F)** Young inflorescence with a terminal and two lateral flowers. **(G)** Flower meristem and two axillary meristems. **(H)** Floral bud elongating with young sepals **(I)** Floral bud with sepals and incipient petal and stamen primordia. **(J)** Floral bud with grown sepals and differentiated petal, stamen, staminodia and carpel primordia. **(K)** Mature floral bud. **(L)** In situ mRNA hybridization of *AqAG.* cl, cauline leaf; fm, floral meristem; lf, lateral flower; la, leaf lamina; lb, leaf base; p, petal; s, sepal; st, stamens; tf, terminal flower; arrowhead indicates carpel primordia; arrows indicate staminodia; asterisks indicate axillary floral buds; dashed line indicates the leaf petiole. Scale bars = 50 μm

Hybridization with the sense *AqFL1* probe showed no signal. An *Aquilegia AGAMOUS (AG)* probe was used as a positive control, and we detected its expression as expected in stamens, staminodia and carpels (Fig. 4.2L).

4.2.c. *AqFL1* regulates leaf morphology, axillary meristem growth and inflorescence height.

To investigate the function of *FUL-like* genes in *Aquilegia* we used the bipartite Tobacco Rattle Virus (TRV) to perform virus induced gene silencing (VIGS) as previously described (Liu et al., 2002; Gould and Kramer, 2007; Pabón-Mora et al. 2012). TRV consists of two RNA molecules, TRV1 and TRV2; the latter includes a cloning site into which a fragment of the target gene can be inserted to induce silencing. We used a TRV2 construct that includes a region of the Anthocyanin Synthase gene (*ANS*) (Gould and Kramer, 2007); down-regulation of *ANS* levels results in a reduction of floral pigmentation, facilitating the screening of plants after infiltration (Kramer et al., 2007; Sharma et al., 2011). We cloned a short fragment of the *AqFL1* gene (Fig.

4.1) into the multiple cloning site of TRV2-ANS to make TRV2-*AqFL1-ANS*. A total of 150 plants with 4-5 true leaves were infiltrated after vernalization with TRV1 and TRV2-*AqFL1-ANS.* In parallel, 100 plants at the same developmental stage were treated with TRV1 and TRV2-*ANS* as a control (control group). Because of technical limitations related to the viability of the viral vectors at low temperatures, plants were vernalized before infiltration, and therefore the function of *FUL-like* genes in this process could not be evaluated.

Silencing of *ANS* in both the control and experimental groups was evident 4-5 weeks after treatment, when plants started flowering. We selected for further screening 45 plants from the target gene group and 40 plants from the control group showing *ANS* silencing. Leaves and cauline leaves of plants showing silencing of *ANS* in both groups were screened using RT-PCR and qRT-PCR for changes in amount of *AqFL1* transcript (Fig. 4.3A). A reduction in transcript abundance was observed in leaves of 34 (75%) of the 45 pre-selected TRV2-*FL1-ANS* plants when compared to pooled samples from TRV2-*ANS* plants. In addition, to confirm reduction of *AqFL1* transcript in floral tissue, a total of 15 TRV2-*FL1-ANS* sepal samples were screened using qRT-PCR. Most samples showed some degree of down-regulation when compared with pooled TRV2-*ANS* sepals (Fig. 4.3B). Plants that show down-regulation of the target gene are hereafter named *aqfl1*. Because *ANS* silencing does not result in any morphological differences other than changes in flower color, the TRV2-ANS treated plants are hereafter designated "wild-type" and used to control for wild type characteristics.

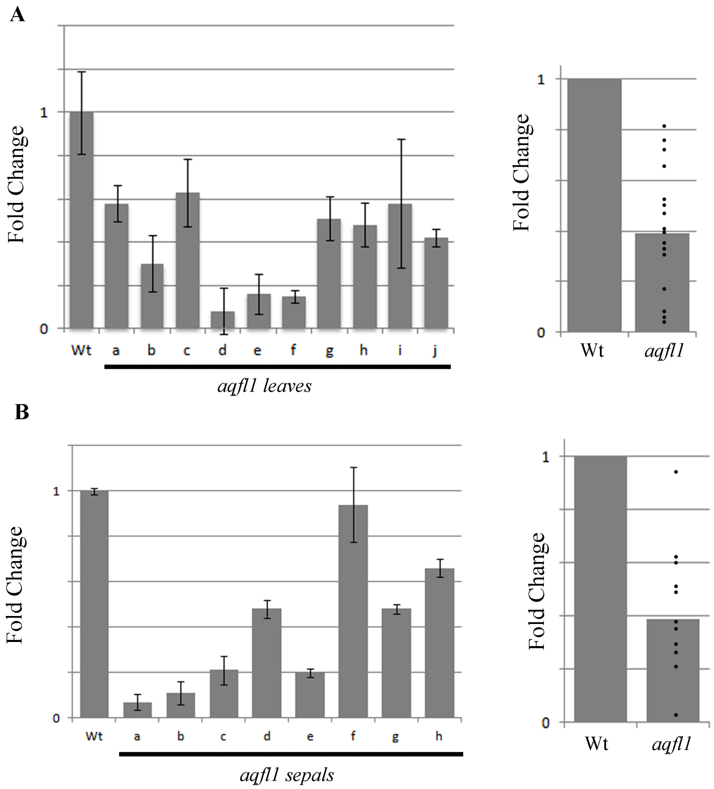


Figure 4.3. Down-regulation of *AqFL1* in VIGS treated plants. **(A)** Left**,** locus-specific qRT-PCR using cDNA prepared from leaves of VIGS-treated plants showing the fold change in *AqFL1* expression relative to pooled wild type leaves in 10 individual *aqfl1*leaves (a-j). Right**,** average silencing of *AqFL1* after repeating the screen in 15 additional leaf samples. Dots indicate fold change in individual samples. **(B)** Left, locus specific qRT-PCR using cDNA prepared from sepals of 8 different VIGS treated plants selected from ones that showed down-regulation in leaves. Right, average silencing of *AqFL1* after repeating the screen in 7 additional samples. Dots indicate fold change in individual samples Error bars show ± SD among three technical replicates. *AqIPP2* was used as the endogenous control.

In contrast to the range of phenotypes associated with other *ap1/ful* mutants, *aqfl1* plants showed no differences in flowering time or defects in floral meristem identity, floral organ identity, or fruit development (Supp. Fig 4.1). However, similarly to other *ap1/ful* mutants, differences in axillary meristem growth and inflorescence size were noticed (Supp. Fig 4.1). Plants down-regulated for *aqfl1* showed outgrowth of axillary buds in the rosette (Supp. Table 4.1); whereas wild type plants (n=30) produced one to two inflorescences (Mean (M)=1.2 ± 1.43), *aqfl1* plants (n=34) produced up to four (M= 1.78 ± 0.88; p< 0.005). In addition, a reduction in the final inflorescence height was also observed. While wild type inflorescence axes often reached over 10 cm (M= 13.13 ± 3.09), *aqfl1* inflorescences often arrested elongation at 2-5 cm (M= 7.69 ± 4.6; p<0.001), resulting in open flowers at the level of the rosette leaves (Fig 4.4B; Supp. Fig. 4.1). To determine whether this stunted growth resulted in fewer flowers per inflorescence, we counted the number of flowers per inflorescence in wild type and *aqfl1* plants and found that whereas wild type inflorescences often bear three to five flowers (M=3.9 ± 0.80), *aqfl1* plants produce a minimum of one and a maximum of four (M= 2.7 ± 1.1; p<0.001). In summary *aqfl1* plants exhibited shorter central inflorescences with fewer flowers, and increased axillary meristem growth resulting in more inflorescences.

Unexpectedly, the most conspicuous abnormality in plants down-regulated for *aqfl1* was a change in leaf morphology (Figs. 4.4A-J; Supp. Table 1). Rosette leaves in 13 *aqfl1* plants (38%) exhibited reduced lamina complexity, often showing two to three leaflets with smooth, entire margins and little lobing (Fig. 4.4D). However they showed no defects in the leaf base or petiole. Cauline leaf morphology (Figs. 4.4E-J) was also abnormal in 29 *aqfl1* plants (85%) which exhibited: 1) increased length of the petiolules particularly in the basal-most but occasionally in the upper-most cauline leaves (Fig. 4.4F, J); 2) a reduction in leaf complexity often showing two to three leaflets with no segments and smooth, entire margins (Figs. 4.4F, H, J); and 3) broadening of the lamina especially in the upper-most cauline leaves (Fig. 4.4J, Suppl. Fig. 4.2).

aqfl1 rosette and cauline leaves with entire margins were compared with wild type rosette and cauline leaves under the SEM to determine whether there was any loss of identity or epidermal abnormalities associated with the different shape. Analysis of epidermal cells showed no abnormalities of *aqfl1* rosette leaves compared to wild type (Figs. 4.4K-N). *aqfl1* cauline leaves (Figs. 4.4O-R) possess normal abaxial epidermis, but the normal smooth rounded cells on the adaxial surface are replaced by papillate cells, more similar to cells of the adaxial surface of the rosette leaf (Figs. 4.4P, R). In addition, leaf clearings show that the number of main vascular traces entering each leaflet is the same in *aqfl1* and wild type plants but their trajectory along the lamina differs. In wild type leaves, vascular traces extend outwards to irrigate the central lobes of each leaflet, whereas in *aqfl1* leaves the vascular traces converge at the tip of the simple leaf/ leaflet (Figs. 4.4S-V). Finally, cross sections of the leaves show that the mid-vein of both the *aqfl1* rosette and cauline leaves is less developed and has fewer vascular cells, when compared to the wild type leaves (Figs. 4.4W-Y). These results show that *AqFL1* plays a role in repression of axillary bud growth, inflorescence height, and leaf morphogenesis.

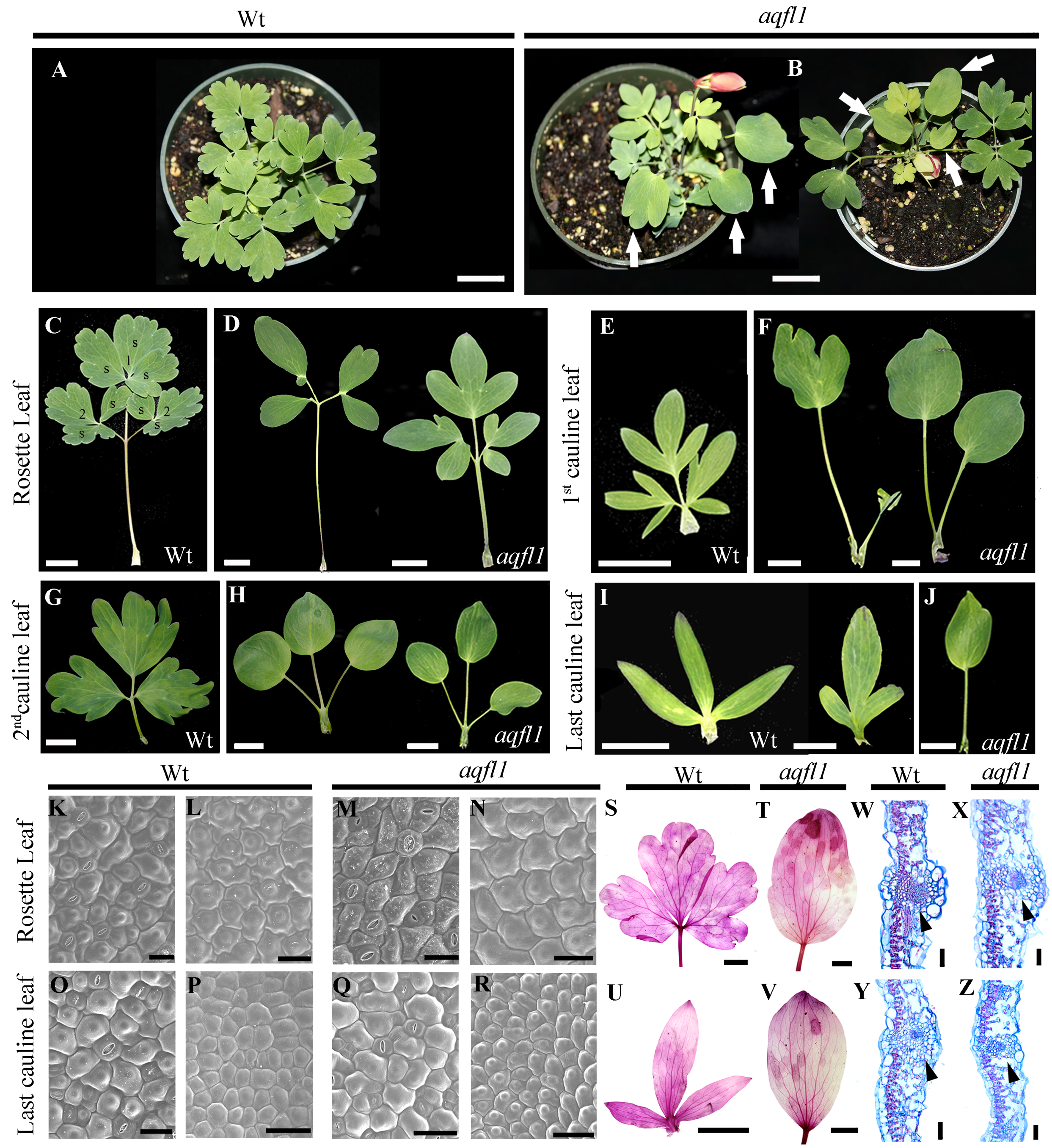


Figure 4.4. Rosette and cauline leaf phenotypes in Aquilegia plants treated with TRV2-ANS-AqFL1.

Top view of **(A)** Wild type and **(B)** *aqfl1* plants. **(C)** and **(D)** Close up of selected **(C)** Wild type and **(D)** *aqfl1* rosette leaves; **(E)** wild type and **(F)** *aqfl1* first cauline leaves; **(G)** wild type and **(H)** *aqfl1* second cauline leaves, and **(I)** wild type and **(J)** *aqfl1* last cauline leaves. **(K)** and **(L)** SEM of the **(K)** abaxial and **(L)** adaxial wild type rosette leaf surface. **(M)** and **(N)** SEM of the **(M)** abaxial and **(N)** adaxial *aqfl1* rosette leaf surface. **(O)** and **(P)** SEM of the **(O)** abaxial and **(P)** adaxial wild type cauline leaf surface. **(Q)** and **(R)** SEM of the **(Q)** abaxial and **(R)** adaxial *aqfl1* cauline leaf surface. (**S**) to **(V)** Clearings of **(S)** wild type and **(T)** *aqfl1* rosette leaves; and **(U)** wild type and **(V)** aqfl1 cauline leaves, a single leaflet is shown in S, T and V. **(W)** to **(Z)** Cross sections of the **(W)** wild type and **(X)** *aqfl1* rosette leaves, and **(Y)** wild type and (Z) aqfl1 cauline leaves. Arrows point to the abnormal leaves in *aqfl1* plants; arrowheads indicate mid vascular bundle. s, segments or secondary leaflets. Scale bars: A,B= 2.5 cm; C-J; S-V= 1 cm; K-R= 30μm;W-Z = 50 μm.

4.2.d. Leaflets and lobes form very early in *Aquilegia* leaf development.

One disadvantage of VIGS is that by the time the abnormal phenotypes can be observed, the early developmental stages at which those abnormalities were initiated have passed, and can be no longer collected for further study. To better understand when changes in leaf morphology in *aqfl1* mutants start occurring, and whether those overlap with *AqFL1* expression, we wanted to investigate in detail the stages of wild type *Aquilegia* leaf development during which leaflets and lobes initiate. Formation of the three main leaflet primordia takes place very early in leaf development (Fig. 4.5A). Leaflets are initiated basipetaly resulting in a slightly larger central leaflet compared to the lateral ones (Fig. 4.5A). Each leaflet primordium further differentiates three main segments (Fig. 4.5B). Leaves elongate without segmenting or lobing for a period of time (Figs. 4.5C, D) until marginal meristematic activity resumes, forming additional lobes on the three main segments of each leaflet (Fig. 4.5E). All leaflets, segments and lobes are formed before petiole and petiolules elongate (Figs. 4.5F,G). The lowermost cauline leaves follow the same pattern as rosette leaves; in contrast, uppermost cauline leaves do not form lobes (Figs. 4.5H, I). In general, the stages at which leaflets and lobes are initiated correspond to stages at which *AqFL1* is expressed. Altogether, the data suggest that *AqFL1* functions at early stages of leaf development promoting leaflet formation, segmentation and lobing in the lamina.

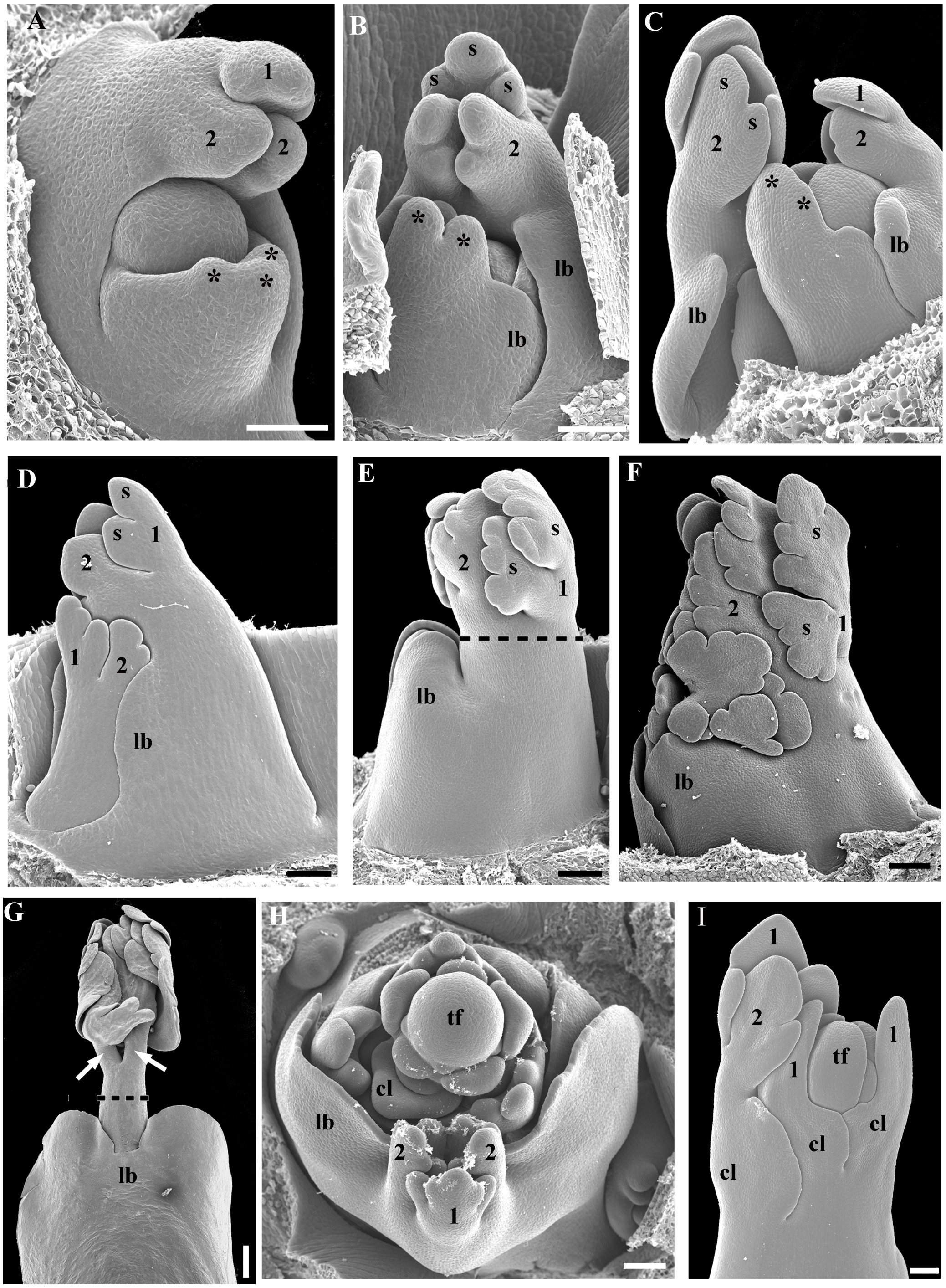


Figure 4.5. Rosette and cauline leaf development in *Aquilegia coerulea*.
(A) Shoot apical meristem flanked by two leaves. **(B)** to **(G)** Leaf development series showing leaflet inception and early lobing of each leaflet, concurrent with the differentiation of the leaf base **(B-D)** and subsequent marginal growth and increase in leaf dissection **(E-G)**. **(H)** Frontal view of the inflorescence showing the terminal flower and the lateral floral primordial still protected by the basal most cauline leaf. **(I)** Elongating inflorescence with three cauline leaves

covering the developing flowers. cl, cauline leaf; lb, leaf base; s, segments in each leaflet; tf, terminal flower; 1 indicates the central leaflet; 2 indicates the lateral leaflets; asterisks indicate the leaflet primordia; arrows indicate petiolules; dashed line indicates the leaf petiole. Scale bars: A-F and H-I= 100 µm; G= 1 mm.

4.2.e. AqFL1 interacts with flowering regulators and floral meristem proteins but not with floral organ identity proteins.

To investigate the overlap in interaction partners between the pre-duplication *Aquilegia* FUL-like protein and AP1 and FUL in *Arabidopsis*, we tested interactions between AqFL1 and the *Aquilegia* MADS-box homologs of the AP1 and FUL protein partners. These include flowering regulators, such as SOC1and AGL24 and floral meristem and floral organ identity genes, such as AGL6, SEP, AP3, PI and AG. *Aquilegia* possesses two AGL24 (Ballerini and Kramer, 2011), four SOC1, SEP, and AP3, two AG, and one PI copies (http://www.phytozome.net; Kramer et al., 2007; Sharma et al., 2011). All genes are co-expressed with *AqFL1*, some during vegetative growth, others during the reproductive transition, and still others in flower development (Supp. Fig 3; Kramer et al., 2007; Ballerini and Kramer, 2011; B. Sharma et al. pers.comm), except for *SOC1.3* and *SOC1.4* whose expression could not be detected in any of the tissues tested for expression of *AqFL1* (Supp. Fig. 4.3). We used a yeast two hybrid (Y2H) system to test protein interactions for the genes that are coexpressed with *AqFL1*. Our results show that AqFL1 is able to homodimerize, and to interact strongly with AqAGL24.1 and AqAGL6. In addition, it interacts weakly with SOC1.1 and SOC1.2 and the four AqSEP homologs. Finally AqFL1 does not interact with AqAGL24.2, AqAP3, AqPI or AqAG homologs (Fig. 4.6; Supp. Fig 4.4) (L. Holappa and E. Kramer personal communication).

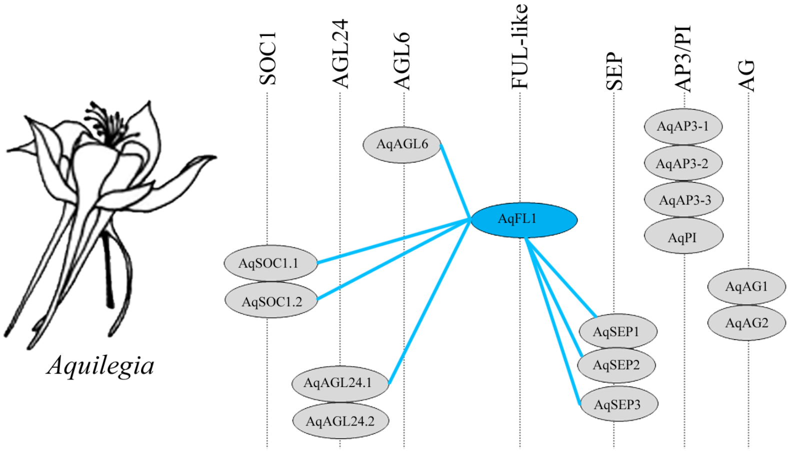


Figure 4.6. Summary of the AqFL1 interactions with other MADS-Box proteins involved in flowering transition, floral meristem and floral organ identity.

4.3. DISCUSSION

4.3.a. The role of *AqFL1* compared to other core and con-core eudicot *AP1/FUL* genes

The results presented here point to a very complex functional evolutionary history of *AP1/FUL* genes that has been shaped by duplication resulting in gene gain and subsequent loss, expression pattern changes, and reshaping of protein interactions, with resulting changes in functional roles. In contrast to previously described roles of *AP1/FUL* genes across angiosperms, *AqFL1* does not appear to play a significant role in floral meristem or organ identity, or in fruit development; down-regulation of this gene produced no floral or fruit defects. This suggests either redundancy with other (MADS box) transcription factors in *Aquilegia* or, alternatively, a decoupling of *AqFL1* from the floral genetic program. This could be due to a loss of key interactions with floral organ identity proteins such as AP3 and PI, as suggested by yeast two-hybrid studies (L. Holappa and E. Kramer pers. comm.), to differential expression of other cofactors, especially *SEPALLATA* homologs, as has been previously postulated (Ballerini and Kramer, 2011), or to both. *AqFL1* has retained the broad expression pattern in leaves, all floral parts, and fruits, that is typical of *euFUL* and other *FUL-like* genes (Fornara et al., 2004; Kim et al., 2005; Preston and Kellogg, 2007; Danilevskaya et al., 2008; Pabón-Mora et al., 2012). These broad expression patterns could have facilitated *euFUL* and *FUL-like* gene redeployment in diverse roles in different taxa through regulatory changes resulting in differential specialization of expression in different taxa.

Functional studies are scarce but support this idea, and suggest that before the monocot-eudicot split, *FUL-like* genes more likely played a role in phase transition, given that *WAP1* is required for flowering in wheat and expression studies suggest this is true for orthologs in other grass species as well (Murai et al., 2003; Trevaskis et al., 2003); Preston and Kellogg 2007). In basal eudicots *FUL-like* genes were recruited to repress axillary meristem growth, both in cauline leaves (in poppies) (Pabón-Mora et al., 2012) and in rosette leaves (in *Aquilegia*). Moreover, the reduction in the number of flowers per inflorescence in *aqfl1* plants suggests an inability to specify or maintain inflorescence meristem identity in the axillary meristems upon the formation of the terminal flower. This is consistent with the overlapping expression of transcripts of *AqAGL24,* a promoter of inflorescence identity, and *AqFL1* in axillary meristems (Ballerini and Kramer, 2011). In addition, this is also correlated with the strong interaction between AqFL1 and AqAGL24.1 observed in yeast two-hybrid studies. Thus the Aquilegia *FUL-like* gene plays an additional role in maintaining inflorescence identity and delaying rather than promoting floral meristem fate.

In addition, our results support the idea that during the diversification of the Ranunculales the function of *FUL-like* genes diverged. Whereas poppy *PapsFL* and *EscaFL* genes play roles in floral meristem, perianth identity and fruit development, cauline leaf development (Pabón-Mora et al, 2012), *AqFL1* may no longer serve these functions, but instead has taken on a role in leaf morphogenesis. Finally, in the transition to core eudicots, *euAP1* genes have been recruited almost exclusively in floral meristem and sepal identity (Irish and Sussex, 1990; Huijser et al., 1992; Berbel et al., 2001; Vrebalov et al., 2002; Benlloch et al., 2006) consistent with the strong interactions between *euAP1* proteins and other floral organ identity factors (Honma and Goto, 2001; Pelaz et al., 2001; Shchennikova et al., 2004; Leseberg et al., 2008); whereas *euFUL* genes have continued to play roles in the transition to reproductive meristems, meristem determinacy, and in fruit development (Gu et al., 1998; Ferrándiz et al., 2000; Muller et al., 2001; Melzer et al., 2008; Jaakola et al., 2010), with considerable variation in their protein partners among species (Immink et al., 2003; Shchennikova et al., 2004; DeFolter et al., 2005; Leseberg et al., 2008).

4.3.b. The role of *AqFL1* in leaf morphogenesis

Our results show that leaf defects in plants with reduced *aqfl1* expression, can be broadly divided into: 1) abnormal growth of cauline leaves associated with vascular defects; 2) a decreased number of leaflets and leaflet segments, as well as reduced marginal lobing in rosette and basal most cauline leaves and 3) longer petiolules in rosette and cauline leaves. Other *ap1/ful* mutants such as *ful* mutants in *Arabidopsis* and double opium poppy *FUL-like* mutants also exhibit overgrown cauline leaves with multiple mid-veins and defects in the distribution of the peripheral vasculature (Gu et al., 1998; Pabón-Mora et al., 2012). Nevertheless, the specific role of *AqFL1* in leaf segmentation and marginal lobing, even in rosette leaves before the reproductive transition, is unique among *AP1/FUL* gene functions reported so far and seems to be acquired after the divergence of Papaveraceae, possibly, in the lineage leading to *Aquilegia*.

In addition, the abnormalities in growth and vascular patterning are suggestive of auxin transport inhibition (Bennett et al., 1998; Mattsson et al., 1999). Likewise, over-development of axillary buds, and dwarfism of *aqfl1* inflorescences can be linked to a block in polar auxin transport (Tsiantis et al., 1999). Although these observations suggest a relationship between normal auxin transport and the wild type role of *FUL-like* genes, no hormonal feedback loop has been previously implicated in the regulation of *FRUITFULL,* or *FUL-like* genes. However, preliminary observations after the ectopic application of hormones to wild type leaves shows changes in wild type leaf morphology. Leaves acquire a more rounded entire margin leaf upon the application of the phytohormone, Indole-3-acetic acid (IAA), and a more dissected and lobed leaf lamina upon the application of the auxin transport inhibitor, N-1-Naphthylphthalamic acid (NPA) (data not shown). Although limited and preliminary, these data suggest that the *aqfl1* leaf phenotype could be due to a disruption of auxin gradients in the leaf, such that auxin gets trapped at the margin and thereby prevents proper development of lobes and teeth.

The regulatory networks regulating leaf shape and dissection have been well studied in core eudicots model species, but less so in basal eudicots. In particular, a conserved role in promoting leaf complexity through reactivation of marginal meristematic activity and leaflet growth in leaf primordia has been attributed to the Class 1 *KNOTTED1-like HOMEOBOX* (*KNOX1*) genes in most angiosperms with compound leaves that have been studied (Bharathan et al., 2002; Hay and Tsiantis, 2010). One exception is the independent recruitment of orthologs of the floral meristem gene *LEAFY* (*LFY*) in place of *KNOX1* in pea and close relatives in the legume "inverted repeat lacking clade" (IRLC) (Hofer et al., 1997; Gourlay et al., 2000; Champagne and Sinha, 2004; Champagne et al., 2007). Regardless of whether the compound leaf uses *KNOX* or *LFY* homologs to promote marginal meristem growth, it appears that a conserved genetic mechanism controlled by the *NAM/CUC3* genes regulates the localized repression of growth that is required for proper compound leaf dissection (Blein et al., 2008). Our results show that *AqFL1* is expressed in the expanding lamina and might function similarly to *KNOX1* and *LFY* homologs, in maintaining marginal meristematic activity, resulting in the formation of leaflets and lobes in early compound leaf development in *Aquilegia*. In addition, because of the striking similarity of leaves in plants that are down-regulated for *aqfl1* and *nam*/*cuc3* in *Aquilegia* (Blein et al., 2008), it is tempting to speculate that both genes, regulate proper compound leaf development and margin lobing in *Aquilegia.* In such scenario, *AqFL1* might be a positive regulator of *NAM/CUC3* genes and vice-versa in a feed-back loop mechanism similar to the one typically described for *KNOX1/LFY* and *NAM/CUC3* genes in other flowering plants (Blein et al., 2008; Efroni et al., 2010; Hay and Tsiantis, 2010).

In conclusion, our functional characterization of *AqFL1* suggests conserved ancestral roles for the *AP1/FUL* gene lineage in the repression of axillary meristems, as well as in the regulation of cauline leaf growth during flowering. In addition, it shows that *AP1/FUL* genes have been recruited for divergent functions in basal eudicots, and have acquired an unexpected new role in leaf morphogenesis in *Aquilegia*. Furthermore, the redundancy or complete loss of function of *FUL-like* genes in the floral genetic program, serves as example of the degree of divergence that can occur across homologs from *Arabidopsis* to more early diverging non-model organisms.

4.4. METHODS

4.4.a. Plant Material and Growth Conditions.

Columbine (*Aquilegia coerulea*) seeds were obtained from Burpee, Warminster, PA, USA. Seeds were germinated, vernalized and grown following Kramer et al. (2007) and Sharma et al. (2011).

4.4.b. Phylogenetic analyses.

FUL-like sequences available for magnoliids and ranunculiids were retrieved from GenBank (accession numbers Supp. Table2). Sequences were aligned in MAFFT and adjusted by hand in BioEdit based on conserved amino acid motifs in the C-terminal domain (N Pabón-Mora et al., unpublished data). Phylogenetic analyses were carried out on nucleotide sequences using parsimony through TNT (Goloboff et al., 2008), using all MIKC domains when available. The tree presented is one of five most parsimonious trees. Bootstraps were calculated in TNT after 2000 replicates.

4.4.c. In Situ Hybridization and Anatomy.

mRNA in situ hybridization and anatomical studies were performed following Pabón-Mora et al. (2012) with the following modifications. Developing apices before and after vernalization were collected from wild-type plants *Aquilegia coerulea* cv. origami growing at the Nolen Greenhouses at The New York Botanical Garden. DNA templates for RNA probe synthesis were obtained by PCR amplification of 400- 600 bp fragments of the K and the C domain of *AqFL1* from cDNA (Fig 4.1; all primers in Supp. Table. 4.3). Slides were hybridized overnight at 52ºC.

4.4.d. qRT-PCR.

To test for levels of *AqFL1* transcript in wild type organs we extracted RNA from cotyledons, a series of dissected organs including the shoot apical meristem (with young leaves not yet expanded) as well as expanding leaves before and after vernalization, and dissected floral organs from pre-anthesis buds as well as fruits. Total RNA was prepared using TRIZOL reagent (Invitrogen) and was subsequently DNase treated. 0.5 µg of total RNA was used for cDNA synthesis with SuperScript III (Invitrogen). The resulting cDNA was diluted 1:10. PCR product was amplified using locus-specific primers designed using Primer Express ™ v 3.0 (Applied Biosystems) (Supplemental Table 2). qRT-PCR was performed as described in Pabon-Mora et al. (2012). *AqIPP2* (isopentyl pyrophosphate: dimethylallyl pyrophosphate isomerase) has been shown to have little quantitative transcriptional variation across different tissues and developmental time points and therefore was used as the constitutive reference transcript (Sharma et al., 2011). The level of *AqFL1* mRNA was analyzed relative to *AqIPP2* using the 2-ΔΔCt method (Livan and Schmittgen, 2001). Standard deviation is reported for three technical replicates of each sample. qRT-PCR reactions were carried out using the 7300 qPCR system and SDS software (Applied Biosystems).

qRT-PCR was also used to confirm down-regulation and to evaluate the reduction of transcript in the down-regulated plants. Levels of *AqFL1* transcript were measured in one to four leaves in each of 25 plants showing morphological alterations and were analyzed relative to pooled samples of wild type leaves. RNA extraction, cDNA preparation, qRTPCR and data analysis were done as above.

4.4.e. RT-PCR.

Expression of *AqFL1, AqSOC1, AqSOC1.2, AqAGL24.1, AqAGL24.2* and *AqAGL6* was assayed in all floral organs, leaves and fruits, using the same cDNA than in the qRT-PCR experiments. Primers used were the same designed for Y2H experiments. Reactions were run for 30-33 cycles at an annealing temperature of 55ºC. *ACTIN* was used as a loading control. PCR was run on a 1% agarose gel stained with ethidium bromide and digitally photographed using a DigiDoc-it® Darkroom UVP equipped with a Cannon PC1089 digital camera.

4.4.f. TRV-VIGS.

A 400 bp fragment of *AqFL1* including the K and C domains of the protein was amplified from floral bud cDNA using primers that added BamHI and XbaI restriction sites to the respective 5’ and 3’ ends of the PCR product (Supp. Fig 4.1). This fragment was cloned into a previously constructed vector carrying a 290 bp fragment of the *Aquilegia ANTHOCYANADIN SYNTHASE*

(*ANS*) gene inserted in the EcoRI and BamHI restriction sites, creating the construct TRV2-*AqFL1-ANS*. Infiltration was done following (Gould and Kramer, 2007).

4.4.g. Yeast Two Hybrid Analyses.

The full coding sequences of *AqFL1, AqSOC1.1, AqSOC1.2, AqAGL24.1 and AqAGL24.2, AqSEP1, AqSEP2, AqSEP3, AqSEP4, AqPI, AqAP3-1, AqAP3-2, AqAP3-3, AqAG1, AqAG2* and *AqAGL6* were cloned from a mixture of leaf and floral cDNA and fused in-frame into the pGADT7 (with an activation domain) and the pGBKT7 (with a binding domain) vectors. Vectors showed no auto-activation. Interactions between each protein and an empty vector were used as controls. Background levels of auto-activation were suppressed by the addition to the growth medium of 3-amino-1,2,4-aminotriazole in a concentration ranging from 2.5 and 30 mM . Interactions were tested for growth on selective medium (SD), lacking Histidine, Triptophan and Leucine (-HWL), lacking Adenine, Triptophan and Leucine (-AWL) and lacking Histidine, Adenine,Triptophan and Leucine (-HAWL).

4.4.h. Scanning Electron Microscopy (SEM).

For scanning electron microcopy studies, tissue was fixed in 70% ethanol, dehydrated through an ethanol series and critical point dried using a Samdri 790 CPD (Rockville, MD). Material was mounted on aluminum stubs with adhesive tabs (Electron Microscopy Sciences), sputter coated with gold palladium in a Hummer 6.2 sputter coater (Anatech, Springfield, VA); and examined and photographed at 10kV in a Jeol JSM-5410 LV Scanning Electron Microscope.

Table 4.1 Summary of phenotypes identified in *aqfl1* plants

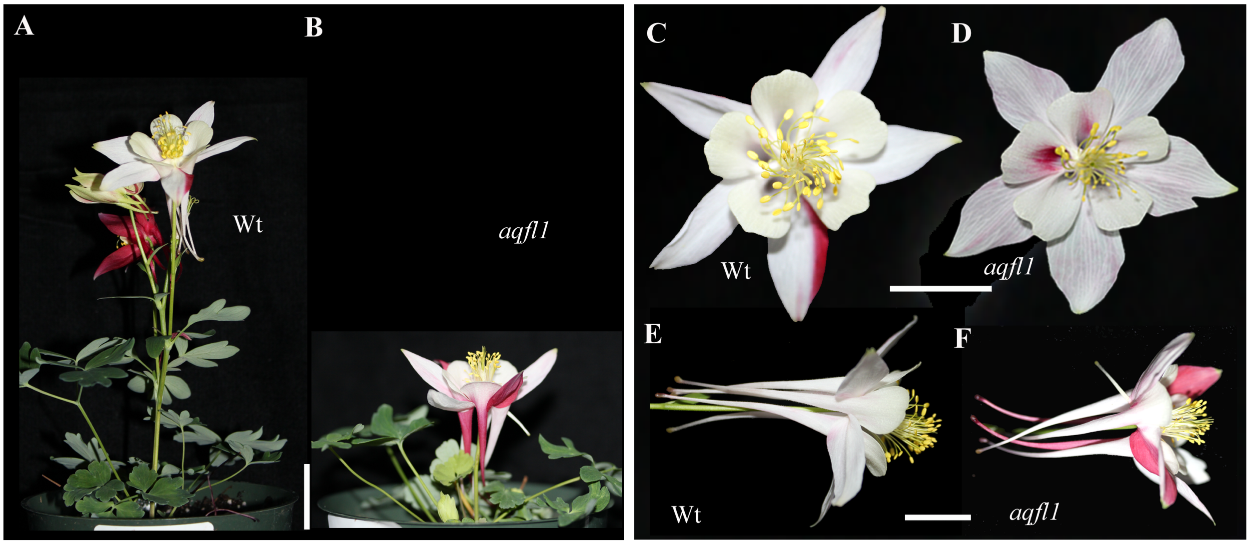
Construct / (effectiveness [a])	Phenotypes	N° plants
TRV2-*ANS*-*AqFL1* (45/150) for *ANS* (34/150) for *AqFL1*	Reduction in rosette leaf complexity	13
	Abnormal cauline leaves	29
	More inflorescences at the axils of rosette leaves (3-5)	11
	Shorter inflorescences (less than 7cm)	22
	Less number of flowers per inflorescence (2 or less)	18

[a] effectiveness: (Total number of plants showing *ANS* silencing/ Total number of plants transformed)

4.5. APPENDIX

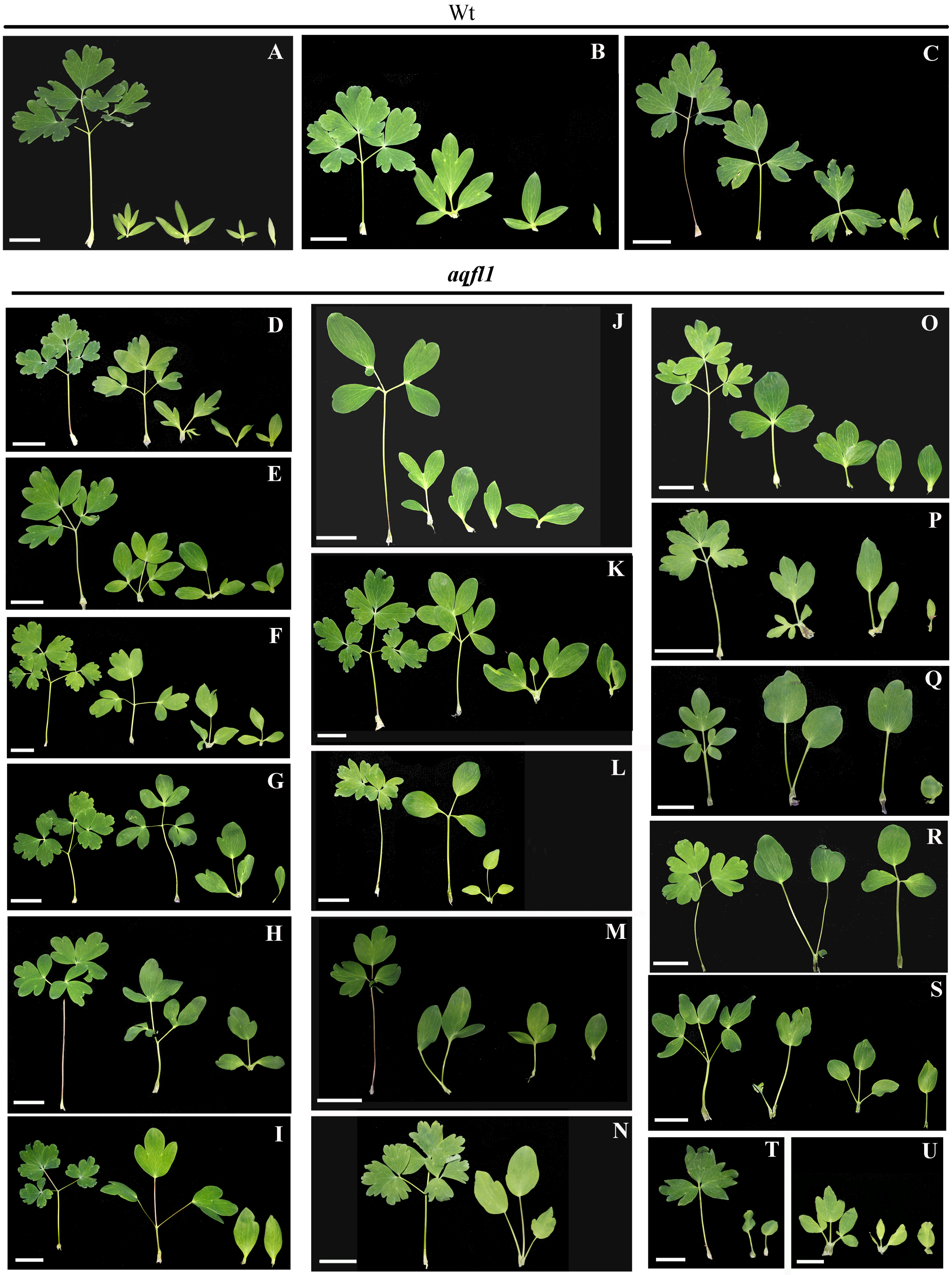
4.5.a. Supplemental Figure 1. Overview of the inflorescences and flowers in wild type and down-regulated *aqfl1* plants.

Wild type **(A)** inflorescence and **(B)** flower. *aqfl1* **(C)** inflorescence and **(D)** flower. Scale bars: 2cm.



4.5.b. Supplemental Figure 2. Summary of the morphological leaf variation in wild type and *aqfl1* plants.

(A) to **(C)** wild type and **(D)** to **(T)** aqfl1 plants. Each panel shows dissected leaves from rosette (furthermost left) to the last cauline (furthermost right) leaf in one individual. aqfl1 panels have been organized from mild phenotypes in the first column (D-I) to stronger phenotypes (J-T). Scale bars: 2cm.


Wt
A
B
C
aqfl1
D
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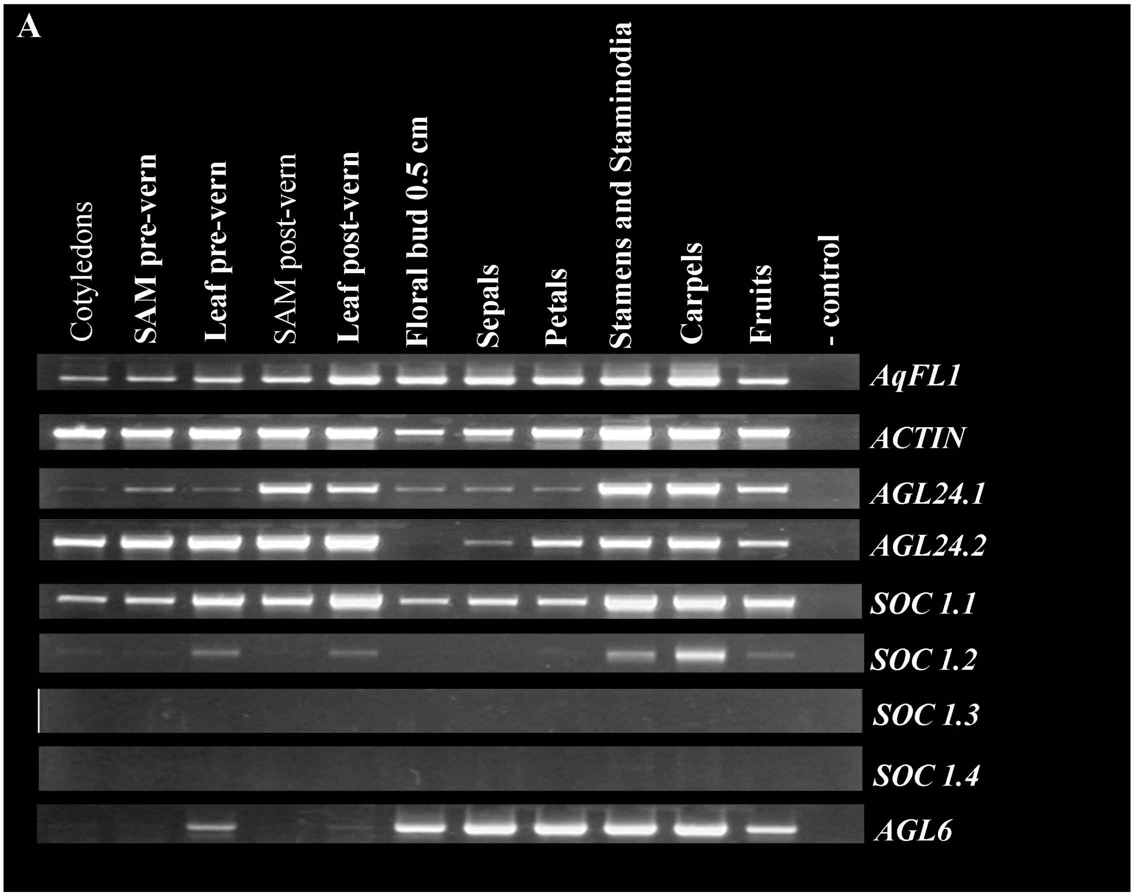
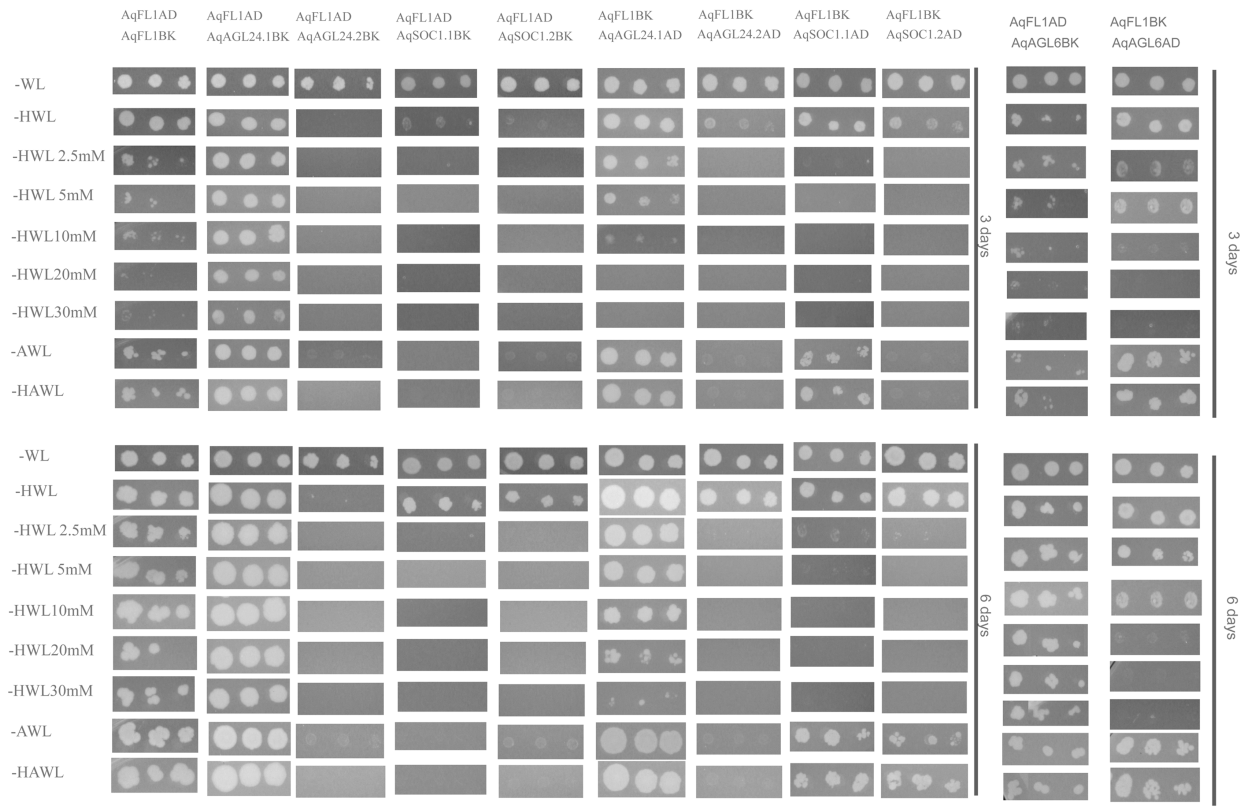
4.5.c. Supplemental Figure 3. RT-PCR results for Flowering Pathway Integrator (FPI) homologs and *AGL6*.

RNA was prepared from pooled organs from one flower of two different plants. Primers were designed specifically for each copy to test for co-expression with AqFL1. All loci were amplified for 30 cycles. SOC1.3 and SOC1.4 are not expressed in the tissues screened.



4.5.d. Supplemental Figure 4. Protein interactions between AqFL1 and Aquilegia Flowering Pathway Integrator (FPI) homologs.

Interactions of AqFL1 were tested with AGL24.1, AGL24.2, SOC1.1, SOC1.2 and AGL6 as determined by growth on selective drop-out medium. Proteins were cloned into both activation domain (AD) and binding domain (BK) vectors. Within each column corresponding to a interacting pair, the three columns of colonies represent a dilution series (10-5, 10-4 and 10-3 colony forming units) of each strain grown on SD medium (- HWL, -AWL and –HAWL). The –HWL medium was supplemented with 2.5 to 30mM 3-amino- 1,2,4 –triazole.



4.5.e. Supplemental Table 1. Sequences and accession numbers used to build a summary *FUL-like* gene tree within Ranunculales.

All sequences were retrieved from NCBI.

Gene name	**Species name**	**Family - Order**	**Accession number**
MagrAP1	*Magnolia grandiflora*	Magnoliaceae - Magnoliales	AY821777
PeamAP1	*Persea Americana*	Lauraceae- Laurales	DQ398019
EuplFL1	*Euptelea pleiosperma*	Eupteleaceae - Ranunculales	DQ656558
EuplFL2			DQ656559
CmFL1	*Chelidonium majus*		AY306144
CmFL2			AY306145
EscaFL1	*Eschscholzia californica*		HM592297
EscaFL2			HM592298
PapsFL1	*Papaver somniferum*		AY306177
PapsFL2			AY306178
PapnFL1	*Papaver nudicaule*		AY306175
SIchFL1	*Sinofranchetia chinensis*	Lardizabalaceae - Ranunculales	DQ656565
SIchFL2			DQ656566
DEinFL1	*Decaisnea insignis*		DQ656556
DEinFL2			DQ656557
AktFL1	*Akebia trifoliate*		AY627632
AktFL2			GU357459
RascFUL1	*Ranunculus sceleratus*	Ranunculaceae - Ranunculales	AB473875
RascFUL2			AB473876
RascFUL3			AB473877
RbFL1	*Ranunculus bulbosus*		AY306179
RbFL2			AY306180
RbFL3			AY306182
RbFL4			AY306183
EsagFL1	*Epimedium saggitatum*	Berberidaceae - Ranunculales	JN590216
DpleFL1	*Dysosma pleiantha*		JN593333
BebeFL	*Berberis bealei*		JN593334
NdomFL1	*Nandina domestica*		JN593335
NdomFL2			JN593338

4.5.f. Supplemental Table 2. Primers used in chapter 4

	Primer name	Primer sequence
In Situ Hybridization	AqFL1 ISH Fwd	GAAGCGAGAAGCACGAGGGCT
	AqFL1 ISH Rev	CTTAATACGACTCACTATAGGGACTGCAATCACACATACCTG
	AqAG ISH Fwd	GGTACAAGAAGGCTTGCACAGACTCTACCAACTCTGGA
	AqAG ISH Rev	CTTAATACGACTCACTATAGGGTTGAAGTGTCGTTTGATCTGAGCGA
Yeast 2 Hybrid	AGL24 Fwd NdeI	**CATATG**GTGAGGGAAAAAATCCAGATC
	AGL24 Rev BamHI	**GGATCC**TAGTGATAACCCCAACCTAAGA
	AGL24.2 Fwd NdeI	**CATATG**GCAAGGGAGAAGATTCAGATC
	AGL24.2 Rev BamHI	**GGATCC**CATTTTCAGCCTTAACTCGAG
	SOC1 Fwd NdeI	**CATATG**GTTAGGGGGAAGACGCAGGTGAAGCGAATC
	SOC1 Rev BamHI	**GGATCC**ACGAGGTTGCAGTAGGCAACCATTAGTAGT
	SOC c Fwd NdeI	**CATATG**GTGAGAGGAAAAACACAGATGAAACGTATA
	SOC c Rev BamHI	**GGATCC**ACATGGCTTTGTCTTTCTTCCTCTATCAGG
	Aq AGL6 EcoRI Fwd	**GAATTC**ATGGGAAGAGGAAGAGTAGAGTTGAAGA
	Aq AGL6 BamHI Rev	**GGATCC**GAGGGCCCAACCCTGGGTGAAGTTGTT
VIGS Insert	AqFL1 EcoRI Fwd	CG**GAATTC**CATGAGTCTTAAAGAACTCC
	AqFL1 XbaI Rev II	CGC**TCTAGA**CACATTAAAATTATCTGACCAT
qRTPCR	AqFL1 Fwd	GGAAATATAAGCACCCCTCATCA
	AqFL1 Rev	CCAAATTACAATCAAAGCAACAACTG
	AqIPP2 Fwd	CAGGTGAAGACGGACTGAAGTTATC
	AqIPP2 Rev	CCAAGACTGGAAAAAGACCACAC

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CHAPTER FIVE

ASSESSING DUPLICATION AND LOSS OF *APETALA1/FRUITFULL* HOMOLOGS IN RANUNCULALES

5.1. INTRODUCCTION

Gene duplication has been posited to provide raw material for evolutionary diversification within organismal lineages, as expansion and functional diversification of gene families provides a mechanism for phenotypic variation (Ohno, 1970; Crow and Wagner, 2006; Roth et al., 2007). Plants provide a useful system in which to study the functional evolution of gene lineages and the effects of gene duplication on organismal diversification. Whole genome duplications and local duplications have occurred frequently and have resulted in genome restructuring across land plants (Blanc et al., 2003; Bowers et al., 2003; Cui et al., 2006; Soltis et al., 2007; Fawcet et al., 2009; Soltis et al., 2010). As a result, gene families and subfamilies have experienced increases and decreases in different taxa (Alvarez-Buylla et al., 2000; Becker and Theissen, 2003; Irish and Litt, 2005; Flagel and Wendel, 2009).

Gene lineage duplications have been particularly well studied in MADS-box transcription factors. MADS-box genes regulate multiple key developmental processes in plants, including the acquisition and maintenance of reproductive meristems as well as the identity of sterile and reproductive floral organs among others (Pelaz et al., 2000; Theissen et al., 2000; Pelaz et al., 2001; Theissen, 2001; Becker and Theissen, 2003; Zik and Irish, 2003; Kim et al., 2004; Kaufmann et al., 2005; Duarte et al., 2006).

MADS-box genes involved in meristem and floral organ identity, also known as the ABCE class genes, are known to have undergone a major duplication event coincident with the diversification of the core eudicots (Kramer et al., 1998; Litt and Irish, 2003; Kramer et al., 2004; Zahn L.M., 2005; Zahn, 2006) as well as taxon-specific duplications before the origin of the core-eudicots (Kramer et al., 2003; Preston and Kellogg, 2006; Drea et al., 2007; Rasmussen et al., 2009; Sharma et al., 2011). MADS-box proteins, particularly the MIKCc plant MADS-box proteins are typically characterized by having a conserved MADS domain that binds DNA at the beginning of the protein (Krizek and Meyerowitz, 1996; Riechmann et al., 1996; Riechmann and Meyerowitz 1997), followed by a relatively variable Intervening domain (I), and a more conserved Keratin-like domain (K), with three regions that form amphipathic helices, important for strength and specificity of protein dimerization (Yang et al., 2003). The C-terminus of the proteins is very variable, but often small motifs can be indentified in this region. For instance some MADS-box genes possess transcription activations or protein modification motifs (Moon et al., 1999; Cho et al., 1999; Yalofsky et al., 2000); in addition, the C-terminus has been associated with the formation of multimeric complexes (Egea-Cortines et al., 1999; Honma and Goto, 2001).

The *AP1/FUL* genes are unique to flowering plants and have undergone several duplication events during angiosperm diversification (Litt and Irish, 2003; Shan et al., 2007). A major duplication correlates with the origin of the core eudicots and resulted in the *euAP1* and *euFUL* gene clades. A second duplication within the *euFUL* clade resulted in the *euFULI* and the *euFULII* clades (Litt and Irish, 2003; Shan et al., 2007). In addition, the first duplication resulted in sequence changes and new motifs at the C-terminus due to a frameshift (Litt and Irish 2003; Vandenbussche et al., 2003), providing euAP1 proteins with two new motifs: a transcription activation (Cho et al., 1999) and a farnesylation motif (Yalofsky et al., 2000). FUL proteins have instead a six hydrophobic amino-acid motif with unknown function. The second duplication within the FUL clade resulted in maintenance of the FUL-motif. The pre core-eudicot proteins are characterized by having a conserved FUL-motif and hence have been named FUL-like proteins (Litt and Irish, 2003). Prior to the core-eudicot duplication other taxon specific duplications also occurred in ranunculiids and monocots (Litt and Irish 2003; Shan et al., 2007), and have been particularly well studied in Poaceae (Preston and Kellogg 2006). However these local duplications have maintained the FUL-motif intact.

The order Ranunculales comprises close to 4500 species in 202 genera, circumscribed in eight families (Wang et al., 2009; APG 2009). Families include the early diverging Eupteleaceae, Fumariaceae and Papaveraceae and the Core Ranunculales formed by the families [[Lardizabalaceae + Circaeasteraceae] + [Menispermaceae [Berberidaceae+ Ranunculaceae]]] . This order includes several key species with short life cycles, mostly herbaceous and susceptible to gene down-regulation by means of virus induced gene silencing, that have emerged as non-core eudicot "model-organisms" for the study of gene evolution and function. These include *Cysticapnos vesicaria* (Fumariaceae) (Hidalgo et al., 2012), *Papaver somniferum, Papaver rhoeas and Eschscholzia californica* (Papaveraceae; Hileman et al., 2005; Wege et al., 2007;

Pabon-Mora unpl. data), and *Aquilegia coerulea* and *Thalictrum dioicum* (Ranunculaceae; Gould and Kramer, 2007; Di Stilio et al., 2010).

The goal of this study is to isolate *FUL-like* genes from Ranunculales and explore their evolution in order to identify gene duplication and losses in the Ranunculiid *FUL-like* genes. In addition, the gene phylogeny will be used to determine whether events in the evolution of the gene lineage are correlated with shifts in morphological characters associated with the role of the genes. Recently, comparative functional data have been generated for Ranunculiid *FUL-like* genes. It has been shown that the two *FUL-like* copies in different species of Papaveraceae have some overlapping and some unique functions; thus, opium poppy and California poppy *FUL-like* genes function in repressing inflorescence branching, determining proper floral meristem and sepal identity, and in promoting proper fruit wall development. However, opium poppy proteins have additional roles including proper cauline leaf development, regulation of flowering time and petal identity (Pabón-Mora et al., 2012). In addition, functional characterization of the single *FUL-like* gene present in the *Aquilegia* (Ranunculaceae) genome shows that it has some similar roles to the poppy *FUL-like* genes and some unique roles among the *AP1/FUL* gene lineage. *Aquilegia FUL-like1* (*AqFL1*) is important for repressing axillary meristem growth and plays a new role in leaf morphogenesis (chapter 4). On the whole, it is evident that *FUL-like* genes have some conserved functions, but have been recruited in different taxa within Ranunculales to play diverse roles.

This research is centered around the following questions: 1) How did characters associated with the role of *FUL-like* genes (leaf shape, inflorescence type and branching degree, identity and persistency of the sepals or fruit morphology and dehiscence) evolve in Ranunculales? 2) Can any of the character states of these morphological characters be correlated with duplications, losses or sequence changes of *FUL-like* genes? 3) How many and when did duplication events and losses of *FUL-like* genes occurred in the Ranunculales? 4) Are there noticeable differences in the relative rates of evolution in different gene clades?.

5.2. RESULTS

In the first part of the results variation of character state on the morphological features associated with the function of *FUL-like* genes are mapped onto a composite species tree, obtained by assembling published trees of the Ranunculales (Hoot et al., 1999; Wang et al., 2009). In the second part of the results Ranunculiid *FUL-like* cloned genes and predicted proteins are described. These sequences are then analyzed and a gene tree is presented to explore duplications and losses of *FUL-like* genes in the Ranunculales. In addition relative rates of evolution are analyzed for all clades and subclades found in the gene tree analyses. Finally, correlations between sequence, morphological characters and function of *FUL-like* genes in Ranunculales are evaluated in the context of the gene phylogenetic information.

5.2.a. Character states of leaf type and margin optimized in Ranunculales

Optimization of the leaf type shows that simple leaves are ancestral in Ranunculales and compound leaves have evolved independently multiple times in Fumariaceae, Papaveraceae, Lardizabalaceae, Berberidaceae and Ranunculaceae. Simple leaves are characteristic of

Eupteleaceae and Menispermaceae, but occasionally also occur in Papaveraceae, Berberidaceae and Ranunculaceae (Fig. 5.1A). Leaf margin varies greatly, and although Eupteleaceae has serrated margin leaves, the optimization favors entire margins for leaves and leaflets as the plesiomorphic condition for the rest of the Ranunculales, lobed and serrated margins have evolved independently multiple times across the order (Fig. 5.1B).

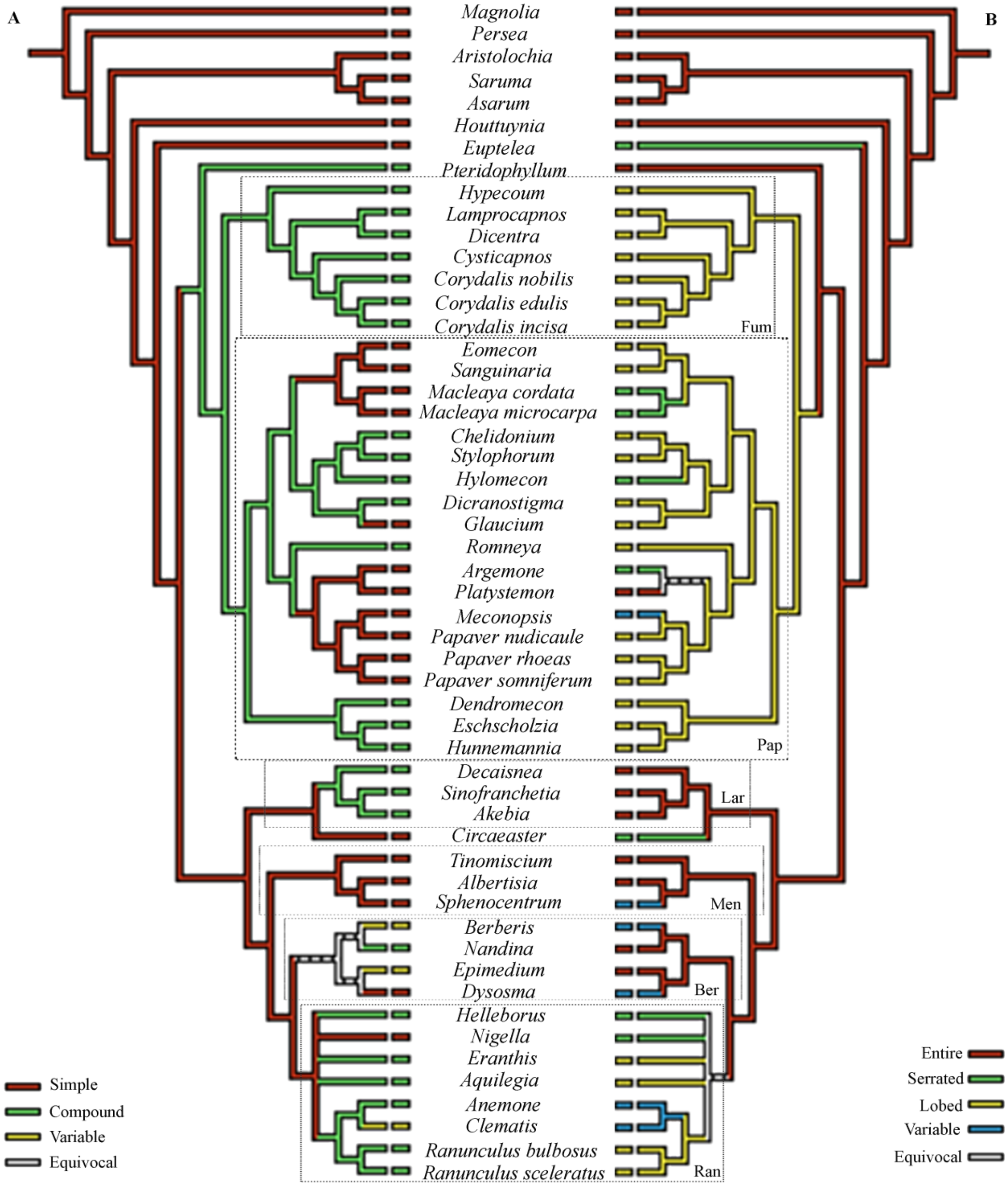


Figure 5.1. Composite phylogeny of the Ranunculales showing optimized character states for leaf shape (A) and leaf margin (B). Dashed boxes indicate different families; Ber= Berberidaceae, Fum= Fumariaceae, Lar= Lardizabalaceae, Men= Menispermaceae, Pap= Papaveraceae, Ran= Ranunculaceae. Phylogeny based on Wang et al., 2009. Outgroup: *Magnolia, Persea, Aristolochia, Saruma, Asarum, Houttuynia* and *Euptelea*. For character sources and states see materials and methods.

5.2.b. Character states of inflorescence morphology optimized in Ranunculales

Optimization of the inflorescence structure shows that racemes are ancestral in Ranunculales and are characteristic of Eupteleaceae and Lardizabalaceae but they have been lost and regained numerous times during the evolution of Ranunculales (Fig. 5.2A). The Papaveraceae (except for Pteridophyllum), Circaeasteraceae and Ranunculaceae are for the most part characterized by cymes, whereas, the Fumariaceae, Menispermaceae and Berberidaceae have taxa with cymes and racemes (Kadereit, 1993; Nickol, 1995; Kessler, 1993). This variation prevents optimization at the node where Papaveraceae + Fumariaceae split from Lardizabalaceae+ Circaeasteraceae or at the base of Menispermaceae + [Ranunculaceae + Berberidaceae]. The number of orders of branches is very variable (Fig. 5.2B). Solitary flowers with no branching occur in Papaveraceae, Menispermaceae, Berberidaceae and Ranunculaceae, but not in Eupteleaceae, Fumariaceae, Circaeasteraceae or Lardizabalaceae. Not surprisingly, branched inflorescences and variation in the numbers of orders of branches occur repeatedly in all families.

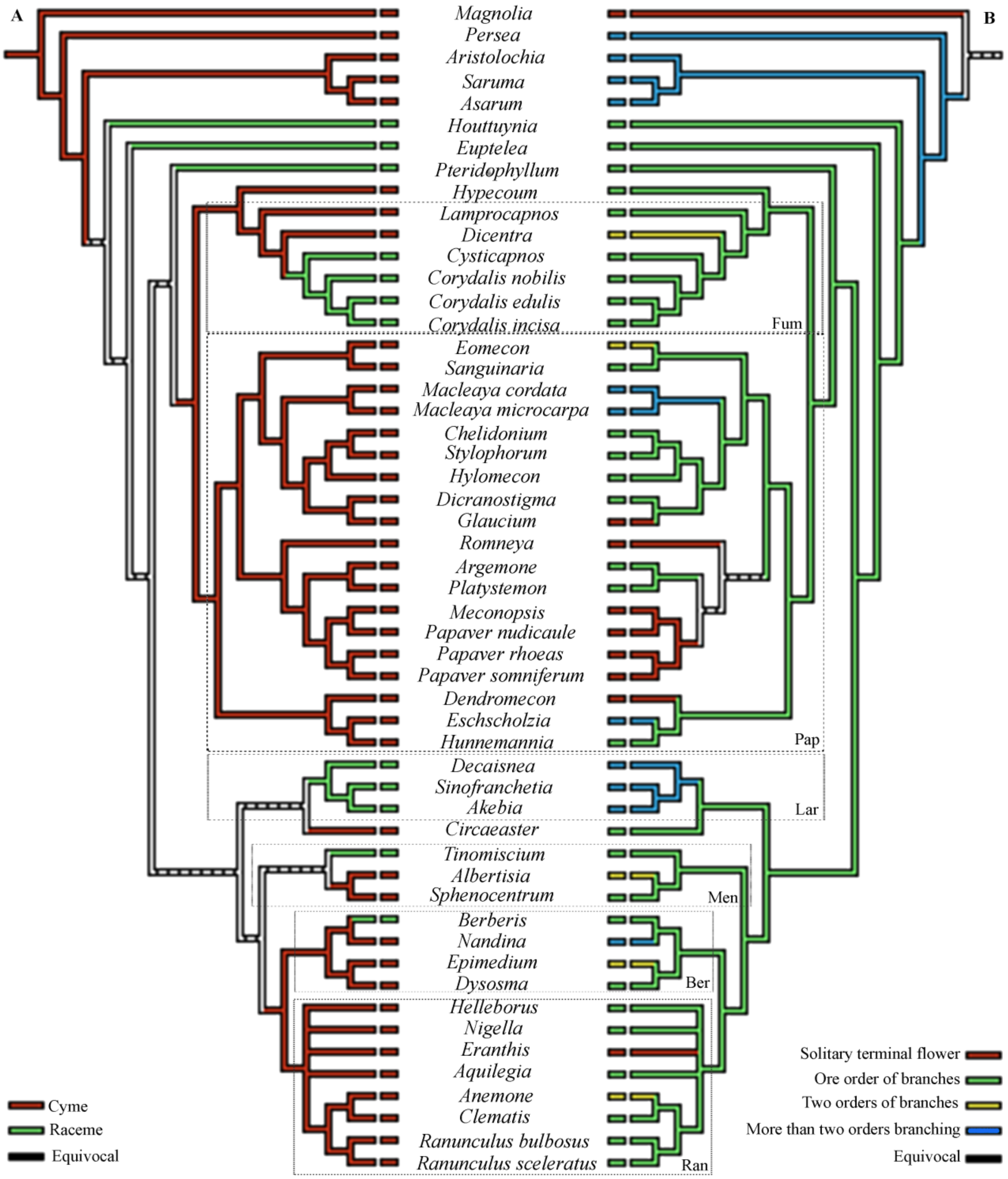


Figure 5.2. Composite phylogeny of the Ranunculales showing optimized character states for inflorescence type (A) and branching degree (B). Dashed boxes indicate different families; Ber= Berberidaceae, Fum= Fumariaceae, Lar= Lardizabalaceae, Men= Menispermaceae, Pap= Papaveraceae, Ran= Ranunculaceae. Phylogeny based on Wang et al., 2009. Outgroup: *Magnolia, Persea, Aristolochia, Saruma, Asarum, Houttuynia* and *Euptelea*. For character sources and states see materials and methods.

5.2.c. Character states of sepal morphology optimized in Ranunculales

Sepal morphology varies greatly across Ranunculales. Sepals can be sepaloid or petaloid (green vs. brightly colored respectively, after Rasmussen et al., 2009), can persist or abscise after anthesis, and can be fused or free in different taxa. Petals can be present or absent and can be large and showy or greatly reduced in size and inconspicuous (Endress, 1995; Rasmussen et al., 2009). Reconstructions of the ancestral sepaloidy or petaloidy of sepals (Fig. 5.3A) is equivocal in the order Ranunculales because the early divergent Eupteleaceae lacks a perianth. However, it is clear that petaloid sepals are ancestral in Fumariaceae, Lardizabalaceae, and Ranunculaceae; whereas sepaloid sepals are ancestral in Papaveraceae, and characteristic of the Circaeasteraceae, although sepaloid sepals also have been independently acquired in at least some members within Berberidaceae (e.g. *Dysosma* and *Epimedium*) and Ranunculaceae (e.g. *Ranunculus*). In addition, sepaloidy or petaloidy of sepals is not correlated with persistence or abscission of sepals after anthesis (Fig. 5.3B). However, some trends can be noticed, for instance most members of the Papaveraceae exhibit deciduous sepals, independent of whether they are sepaloid or petaloid. Sepal persistence is ancestral in Lardizabalaceae + Circaeasteraceae and Ranunculaceae, but is equivocal in Berberidaceae, where sepals can be persistent (e.g. *Epimedium*) or can be shed (e.g. *Dysosma*, *Berberis*, *Nandina*) independent of whether they are sepaloid or petaloid.

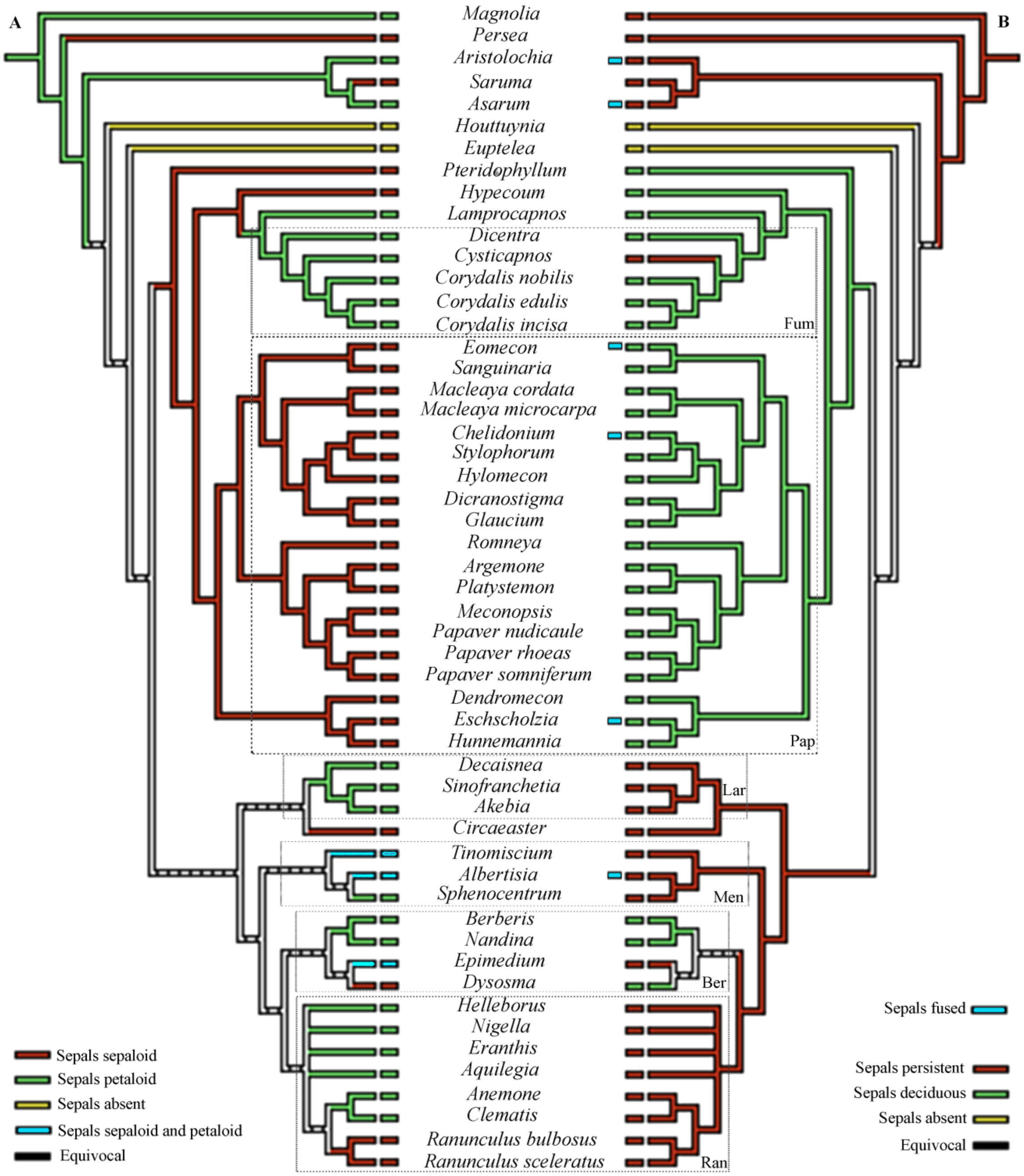


Figure 5.3. Composite phylogeny of the Ranunculales showing optimized character states for sepal morphology (A) and persistence (B). Degree of sepal fusion has been additionally mapped on B. Dashed boxes indicate different families; Ber= Berberidaceae, Fum= Fumariaceae, Lar= Lardizabalaceae, Men= Menispermaceae, Pap= Papaveraceae, Ran= Ranunculaceae.

Two other variations in perianth morphology occur, although less frequently. 1) Sepals are ancestrally free but become (congenitally) fused three times independently within Papaveraceae in *Chelidonium*, *Eomecon* and *Eschscholzia.* The combination petaloid and fused sepals only occurs rarely in Ranunculales. One example is the genus *Albertisia* (Menispermaceae) that has three whorls of sepals, the outer two sepaloid and free, the innermost petaloid and tubular (Kessler, 1993). 2) There is variation in the number of whorls occupied by sepals and/or petals across different taxa. The ancestral condition, after the diversification of Eupteleaceae, is one whorl of sepals, but the number of whorls of petals is equivocal. For instance, Papaveraceae often have one whorl of sepaloid sepals and two whorls of showy petals (except in *Bocconia* and *Macleaya,* which lack petals). Members of the Ranunculaceae have for the most part one whorl of petaloid sepals (or sepaloid sepals like in Ranunculus) and one whorl of petaloid petals (except in *Thalictrum* and *Caltha* where petals are lacking), whereas some Berberidaceae, like *Epimedium* have two whorls of sepals, the outermost sepaloid, the innermost petaloid, in addition to an additional innermost whorl of showy and small petals.

5.2.d. Character states of gynoecium and fruit morphology optimized in Ranunculales

Gynoecium and fruit type vary greatly in Ranunculales. The ancestral condition for degree of carpel fusion is equivocal, however different morphologies have become fixed in different families. Whereas Fumariaceae and Papaveraceae have a syncarpous gynoecium, the apocarpic condition is common in Eupteleaceae and is a synapomorphy for [Lardizabalacaeae [Menispermaceae[Berberidaceae + Ranunculaceae]]]. Members of the Ranunculaceae have predominantly an apocarpous gynoecium, but the carpels are frequently described as being connate to different degrees. Berberidaceae is unique in that most members of the family possess a unicarpellate gynoecium, derived from a multicarpellate condition (Fig. 5.4A). In terms of fruit type, the ancestral condition is the presence of dry fruits, but within that category indehiscent samaras are predominant in Eupteleaceae, whereas longitudinally dehiscent fruits, whether derived from a syncarpic gynoecium (capsules) or an apocarpic gynoecium (follicles) are plesiomorphic for the rest of the families in the order. Indehiscent berries have been independently acquired in Lardizabalaceae and Berberidaceae, and drupes, also indehiscent, are characteristic of the Menispermaceae (Figs. 5.4B). Achenes have been independently acquired in Circaeasteraceae and some Ranunculaceae. Capsules are typical in Fumariaceae, Papaveraceae and Ranunculaceae (Fig. 5.4B).

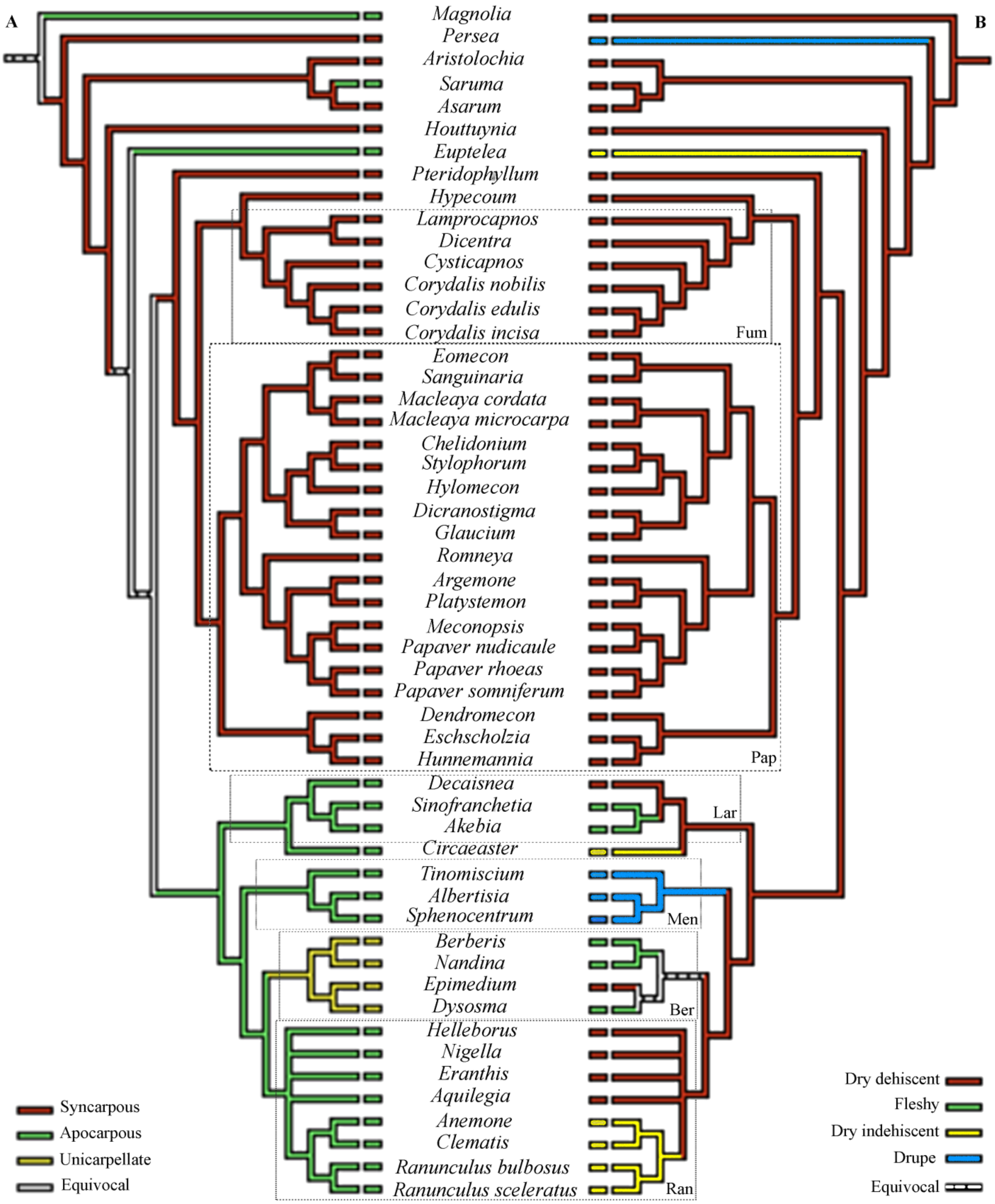


Figure 5.4. Composite phylogeny of the Ranunculales showing optimized character states for carpel (A) and fruit type (B). Dashed boxes indicate different families; Ber= Berberidaceae, Fum= Fumariaceae, Lar= Lardizabalaceae, Men= Menispermaceae, Pap= Papaveraceae, Ran= Ranunculaceae. The category dry indehiscent fruits includes achenes (typical in

Circaeasteraceae and Ranunculaceae) as well as samaras characteristic of the Eupteleaceae. Dry dehiscent fruits include capsules (in Fumariaceae and Papaveraceae, some members of Lardizabalaceae and Berberidaceae) as well as follicles (characteristic of Ranunculaceae).

5.2.e. *FUL-like* gene cloning in Ranunculales

In order to better understand the evolutionary history of *FUL-like* genes in Ranunculales a dataset consisting of a total of 87 gene sequences from this lineage (Table 5.1) from Eupteleaceae, Papaveraceae, Fumariaceae, Lardizabalaceae, Berberidaceae and Ranunculaceae was compiled. Sequences were obtained by degenerate PCR (51 new *FUL-like* genes from 27 taxa) and from public databases (7 *FUL-like* genes from 4 taxa un-annotated available in http://www.phytometasyn.ca , and 29 already identified *FUL-like* genes from 11 taxa from Genbank) belonging to the families Eupteleaceae, Fumariaceae, Papaveraceae, Lardizabalaceae, Berberidaceae and Ranunculaceae as well as to the outgroup basal angiosperm and monocot families Magnoliaceae, Lauraceae, Saururaceae and Aristolochiaceae and Poaceae. Sequences from Menispermaceae and Circeasteraceae were not included due to limited material availability.

Clones that were recovered with degenerate primers either span the entire coding sequence or are missing 10-20 AA from the start of the MADS domain but include most of the MADS and the IKC domains. The alignment includes 60 AA in the MADS domain, 35-40 AA in the I domain, 70-75 AA in the K domain and 90 AA in the C terminal domain. The MADS domain is highly similar (over 90% amino-acid sequence similarity) so sequence similarity was only calculated for the IKC domains. Among Ranunculales paralogous genes sequence similarity ranges from 52 to 95%, and the variation in sequence similarity between outgroup and ingroup ranges from 50-

75%.. All loci have, in the C-terminal portion of their proteins, the FUL-like motif identified in other previously sequenced FUL-like and euFUL proteins (Litt and Irish, 2003; Preston and Kellogg, 2006; Shan et al., 2007). Alignment of the predicted amino acid sequences of the entire dataset reveals a high degree of conservation in the M, I and K regions until position 184. Most plant MADS proteins have three putative amphipathic α-helices with heptad repeats (**a**bc**d**efg)$_n$, in which **a** and **d** positions are occupied by hydrophobic amino-acids. Putative α-helices are found in the K domain (referred to as K1-K3) and have been postulated to be important for dimerization (Yang et al., 2003; Yang and Jack, 2004).

In the putative amphipathic α-helices of Ranunculiid FUL-like proteins K1 (amino-acids 97-110), K2 (amino-acids 121-143) and K3 (amino-acids 152-258) have the expected hydrophobic amino acids in **a** and **d** in the heptad repeats (**a**bc**d**efg)$_{n..}$ Notably within K1, positions 98 (E), 101 (K), 103 (K), 105 (K), 107 (E) (except for RocoFL2, ArmeFL4), and 110 (Q) (except for MacoFL3, MacoFL4), and within K2 positions 118 (G), 127 (K), 128 (E), 133 (E), 134 (Q), 135 (Q), 137 (D) (except for ArmeFL3), are conserved in all Ranunculales and outgroup FUL-like predicted protein sequences (Suppl. Fig. 5.1). Sequence variation increases at the beginning of the C-domain after the hydrophobic amino acid located in position 184. The C-terminal domain is more variable but three regions of high similarity unique to Ranunculiids can be identified: 1) a region of polar uncharged amino acids (QNS), particularly glutamine (Q), which is encoded by tandem repeats between positions 190-230 in the alignment; 2) a predominantly hydrophobic motif at positions 225-256, with the sequence GQNS-P/LS/TFLLSQSLP-SLNI, conserved in all sequences with the exception of a group of sequences in Ranunculaceae, and 3) a negatively charged region rich in glutamic acid (E) before the conserved FUL-motif LMPPWML (Fig.5.5).

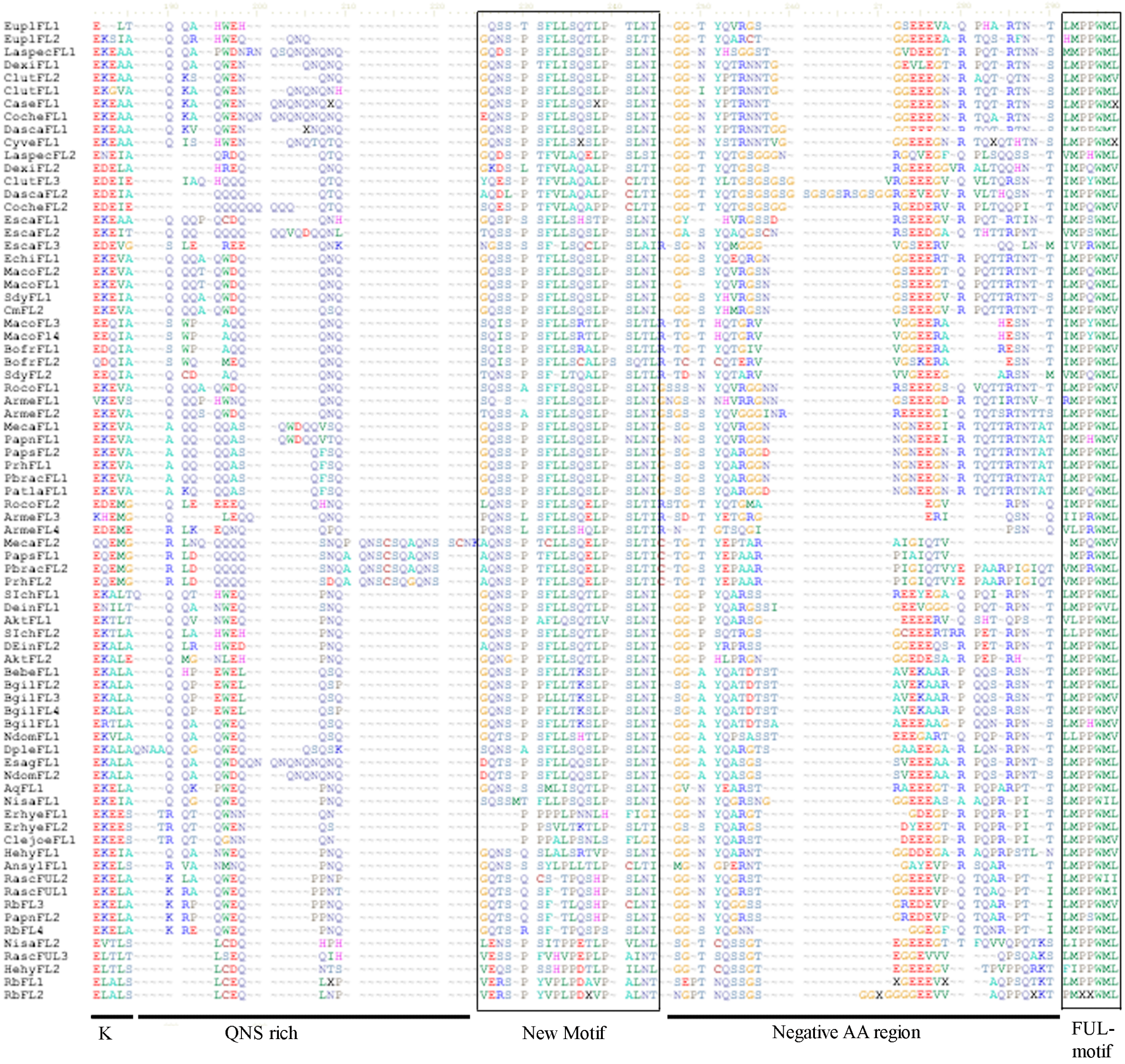


Figure 5.5. Sequence alignment of the end of the K domain (K) and the complete C-terminal domain of Ranunculiid FUL-like proteins, showing a region rich in glutamine (Q), asparagine (N), serine (S), labeled as the QNS rich zone, followed by the conserved hydrophobic motif newly identified (boxed), a region negatively charged and rich in glutamic acid (E) and the FUL-motif (boxed) near to the end of the protein typical of FUL-like and euFUL proteins. CmFL1 was excluded from the alignment because is the only sequence that has an additional insertion in the “new-motif” with 8 additional AA in between positions 229-236.

5.2.f. Gene duplication and loss of *FUL-like* genes in Ranunculales

Parsimony analysis produced three shortest trees with a length (L) of 5686 steps, consistency index (CI)= 0.26 and retention index (RI)= 0.66. A total of 957 characters were included in the matrix, of which 62% (587) were parsimony informative and 38% (370) were uninformative (Fig. 5.6). The three most parsimonius trees had conflicted topologies in the positions of the two Lardizabalaceae *FUL-like* clades , that collapse in a politomy in the parimony strict consensus tree with the (Fumariaceae+ Papaveraceae *FUL-like 1* clade) and the (Berberidaceae + Ranunculaceae *FUL-like 1* clade). However, the strict consensus parsimony tree (Fig. 5.6) is similar to the best Maximum Likelihood tree (Fig. 5.7), both recover two clades of *FUL-like* genes in Ranunculales, here named Ran *FUL-like1* and Ran *FUL-like2*, which diverged before the diversification of all Ranunculales. Bootstrap support for Ran *FUL-like1* and Ran *FUL-like2* clades is low (<50 for *FUL1* and 60 for *FUL2*), however within each clade gene copies from the same family are grouped together with strong support, and the relationships among gene clades are consistent with the phylogenetic relationships of the sampled taxa. The two sequences of Eupteleceae fall in the *RanFUL-1* clade; similarly, the two clades of *FUL-like* genes were recovered from the Lardizabalaceae belong to the *RanFUL-like1* clade. *RanFUL-like2* sequences were also not recovered from Berberidaceae; sequences belonging to the *RanFUL-like2* clade were only recovered from Fumariaceae, Papaveraceae and Ranunculaceae. In addition several amino acids were found that are synapomorphies for *Ran FUL-like1* and *Ran FUL-like2* as well as for taxon specific subclades (Fig. 5.8).

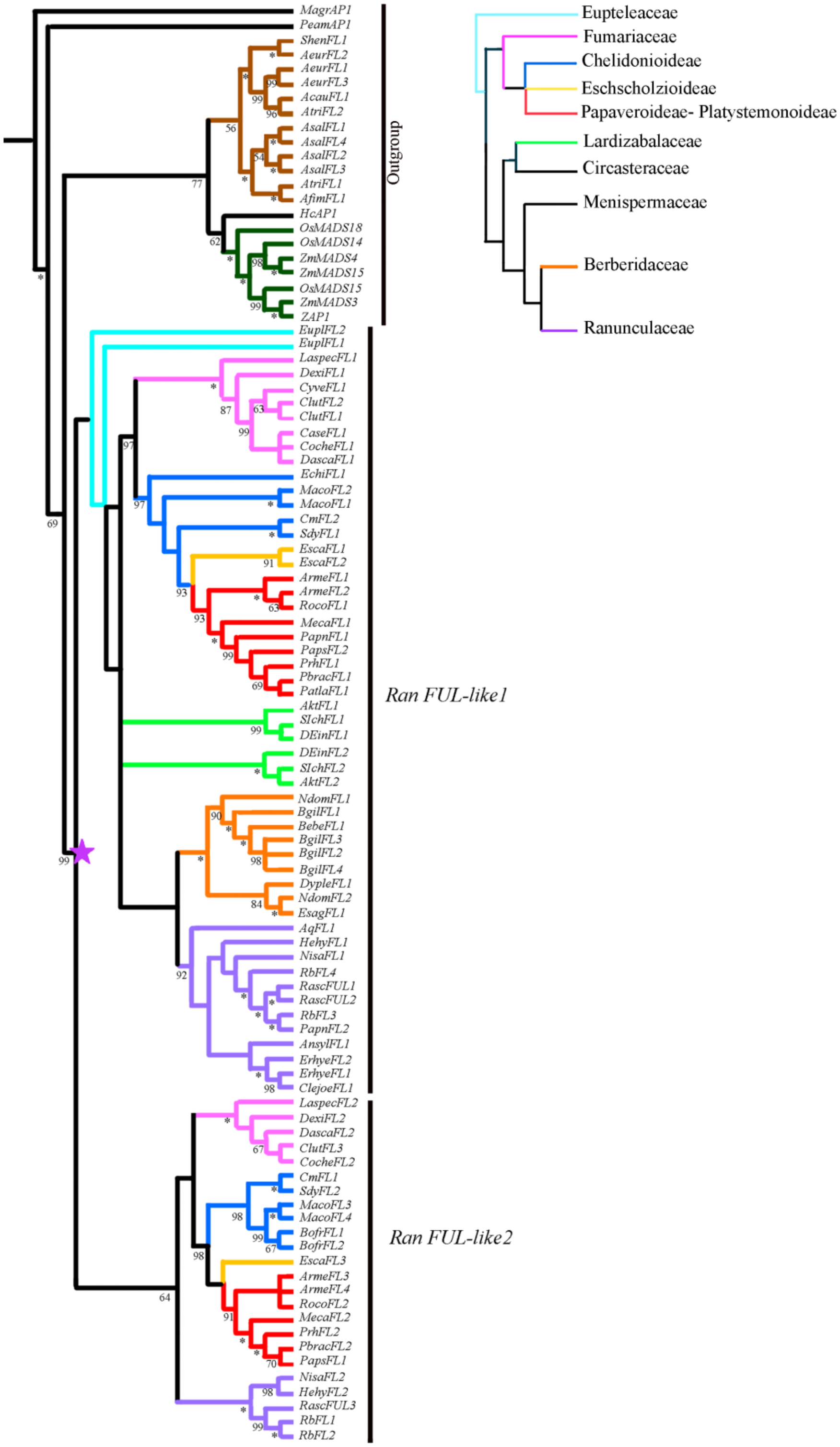

MagrAP1
PeamAP1
ShenFL1
AeurFL2
AeurFL1
AeurFL3
AcauFL1
AtriFL2
AsalFL1
AsalFL4
AsalFL2
AsalFL3
AtriFL1
AfimFL1
HcAP1
OsMADS18
OsMADS14
ZmMADS4
ZmMADS15
OsMADS15
ZmMADS3
ZAP1
Outgroup
EuplFL2
EuplFL1
LaspecFL1
DexiFL1
CyveFL1
ClutFL2
ClutFL1
CaseFL1
CocheFL1
DascaFL1
EchiFL1
MacoFL2
MacoFL1
CmFL2
SdyFL1
EscaFL1
EscaFL2
ArmeFL1
ArmeFL2
RocoFL1
MecaFL1
PapnFL1
PapsFL2
PrhFL1
PbracFL1
PatlaFL1
AktFL1
SlchFL1
DEinFL1
DEinFL2
SlchFL2
AktFL2
NdomFL1
BgilFL1
BebeFL1
BgilFL3
BgilFL2
BgilFL4
DypleFL1
NdomFL2
EsagFL1
AqFL1
HehyFL1
NisaFL1
RbFL4
RascFUL1
RascFUL2
RbFL3
PapnFL2
AnsylFL1
ErhyeFL2
ErhyeFL1
ClejoeFL1
Ran FUL-like1
LaspecFL2
DexiFL2
DascaFL2
ClutFL3
CocheFL2
CmFL1
SdyFL2
MacoFL3
MacoFL4
BofrFL1
BofrFL2
EscaFL3
ArmeFL3
ArmeFL4
RocoFL2
MecaFL2
PrhFL2
PbracFL2
PapsFL1
NisaFL2
HehyFL2
RascFUL3
RbFL1
RbFL2
Ran FUL-like2
Eupteleaceae
Fumariaceae
Chelidonioideae
Eschscholzioideae
Papaveroideae- Platystemonoideae
Lardizabalaceae
Circasteraceae
Menispermaceae
Berberidaceae
Ranunculaceae

Figure 5.6. Strict consensus parsimony tree of *FUL-like* genes in Ranunculales. Bootstrap values (above 50%) are placed at nodes. Asterisks indicate bootstrap values of 100. Star points to the duplication event resulting in the two clades Ran*FUL-like1* and Ran*FUL-like2*. Branch colors indicate plant families as per family tree in the right. Outgroup includes: Magnoliales in black, Piperales in brown and grasses in dark green.

Figure 5.7. Best Maximum Likelihood Tree of *FUL-like* genes in Ranunculales. Topology is identical to the parsimony tree. Bootstrap values (above 50%) are placed at nodes. Asterisks indicate bootstrap values of 100. Star indicate inferred gene duplication event. Branch colors indicate plant families as per Figure 6.

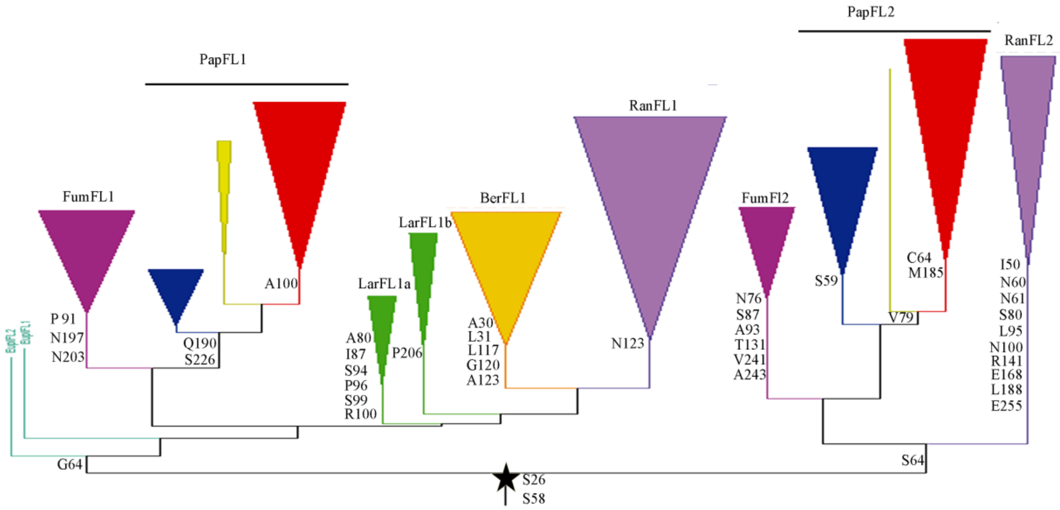


Figure 5.8. Diagnostic amino acid characters of the FUL-like proteins in Ranunculales. The star points to the duplication event

5.2.g. Relative rates of evolution of *FUL-like* genes in Ranunculales

I wanted to test whether any clade or subclade within Ranunculiid *FUL-like* genes is undergoing different rates of evolution, when compared to the rest of the sequences in the tree. Differential rates of evolution can be correlated with different I used the likelihood ratio test of whether the two (or more) clades descended from a particular node are evolving at the same constant rate. This ML-based approach to calculate relative rates of nucleotide evolution as implemented by software package R8s tests the hypothesis that a model with one constant rate for a clade fits the data as well as a two or more different constant rates for subclades comprising the entire clade. Using this approach, I calculated relative rates for outgroup grass *FUL-like* clade and Piperales *FUL-like* clade as well as ingroup clades: *FumFUL-like1*, *PapFUL-like1*, *LarFUL-like1a*, *LarFUL-like1b*, *BerFUL-like1*, *RanFUL-like1*, *FumFUL-like2*, *PapFUL-like2*, *RanFUL-like2* as well as Ranunculales *FUL-like1* and Ranunculales *FUL-like2*. I found that there were no statistically significant differences in the rate of evolution of sequences in the any of the clades or subclades ($p>0.05$), neither were there differences between the outgroup and the Ranunculiid *FUL-like* genes.

5.2.h. Correlating sequences changes and morphological changes

I wanted to investigate whether *FUL-like* sequence changes and specific motifs in the C-domain, have any correlation with changes in inflorescence types, degree of branching, leaf type, sepal morphology, or carpel and fruit type across the order. To do that sequences were organized according to character state in the alignment and variable regions across sequences were inspected for diagnostic aminoacids or motifs that would correlate with their groupings. No clear correlation in sequence was found with any of the morphological characters states explored.

5.3. DISCUSSION

5.3.a. Identification of conserved motifs in the C-terminus of Ranunculiid *FUL-like* genes

I have shown that Ranunculiid FUL-like proteins have an additional conserved motif rich in polar and hydrophobic amino acids GQNS-P/LS/TFLLSQSLP-SLNI upstream of the previously identified FUL-like motif. This motif is present in all Ranunculiid FUL-like genes except for CmFL1 that has a 8AA insertion in the middle of this motif, and is slightly more variable in sequences belonging to the Ranunculaceae *FUL-like 2* clade. In addition, all Ranunculiid FUL-like proteins possess at the beginning of the C terminal domain, glutamine rich segments carrying from 3 to 9 consecutive glutamines (Q) and 3-4 non-consecutive glutamines. Consecutive glutamine-rich motifs are also found in grass FUL-like proteins (Preston and Kellogg, 2006). Glutamine-rich domains in plants have been known to behave as transcription activation domains (Gerber et al., 1994; Schwechheimer et al., 1998); thus, FUL-like proteins could potentially have a similar transcription activation activity to the one that has been normally attributed to euAP1 proteins (Cho et al., 1999). However, AqFL1 (with 2 consecutive and 2 non-consecutive Q), PapsFL1 and PapsFL2 (both with 4 consecutive Q) have not been shown to auto-activate in yeast systems so far. In addition, glutamine repeats in eukaryotes have been hypothesized to behave as "polar zippers" in protein-protein interactions (Perutz et al., 1994; Michleitsch and Weissman, 2000), thus these regions might allow variation in the strength and specificity of FUL-like protein interactions. This variation at the beginning of the C-terminal domain is often specific among paralogous genes within a family and it might be important for functional divergence of *FUL-like* genes in different families (Supp. Fig 5.2). This is consistent with observations by Moon (1999) on rice proteins, in which the 14 amino acids downstream of the K domain may enhance or stabilize protein interactions, as shown by higher *LacZ* activity in a yeast system in the presence of this sequence fragment.

5.3.b. *FUL-like* genes underwent duplication early in the diversification of the Ranunculales at the same time as *AP3* gene lineage duplications

This analysis recovered a duplication in the Ranunculiid *FUL-like* genes before the origin of the order Ranunculales. The position of the EUplFL1 and EUplFL2 in the Ranunuculiid *FUL-like1* clade suggests that these two sequences have diverged little or that the Eupteleaeceae lost its *Ran FUL-like2* members. Additionally, whereas *RanFUL-like1* genes are found in all the families of the order so far sampled, *RanFUL-like2* genes were only identified in Fumariaceae, Papaveraceae and Ranunculaceae. There are several possibilities that explain this: first, our primers may preferentially amplify *RanFUL-like1* vs. *RanFUL-like2* genes; second, *RanFUL-like2* genes may not be expressed in leaf or floral tissue and therefore have not been amplified in our work or in previous work (Shan et al., 2007); or third, *RanFUL-like2* genes became non-functional and are not expressed, or were lost in the Eupteleaceae, Lardizabalaceae and Berberidaceae. None of these hypotheses can be rejected at this time, but I favor the second and the third over the first one, after subsequent attempts of degenerate PCR amplification with multiple primers specifically designed to amplify *RanFUL-like2* copies from Berberidaceae and extensive database search.

Gene duplicates from other floral gene lineages such as *AP3*, *AG* and *SEP* have also been identified in the Ranunculales (Kramer et al., 2003; Kramer et al., 2004; Rasmussen et al., 2009; Liu et al., 2010). Such duplications have generated three AP3 and two SEP and AG Ranunculiid gene clades. The fact that several floral gene lineages duplicated early during the diversification of the Ranunculales is in agreement with a postulated ancient genome duplication in basal eudicots (Cui et al., 2006).

5.3.c. Differences in function among orthologous *FUL-like* genes are not associated with different rates of evolution or expression patterns but might be with a result of differences in protein partners.

I hypothesized that differences in function might be associated with differences in nucleotide rates of evolution, but this is not the case. One possibility that remains to be tested is whether *RanFUL-1* and *Ran FUL-2* and subclades within are undergoing different selective pressure at the level of amino-acid in terms of different rates of synonymous vs. non-synonymous changes.

Differences in function could potentially be attributed to differences in expression patterns. Expression of *FUL-like* genes reported in Ranunculiids and other basal eudicots are broad often including vegetative, inflorescence and floral meristems, leaves, all floral organs and fruits (Wu et al. 2007; Liu et al., 2010; Pabón-Mora et al, 2012;). However, these expression patterns do not show an explicit correlation with the functions that have been identified for *FUL-like* genes. For instance despite their similar expression patterns, *Aquilegia FUL-like* genes have taken on different functions when compared to poppy *FUL-like* genes. *FUL-like* genes in *Aquilegia* play a role in leaf morphogenesis (chapter 4), whereas, Papaveraceae *FUL-like* genes play a role in axillary meristem growth, floral meristem and sepal and petal identity, and fruit development. In addition, *FUL-like* genes are also often expressed in stamen and carpel primordia, but down-regulated plants do not show any defects in stamen or carpel identity suggesting redundancy with other transcription factors. In general, these results point to broad, conserved *FUL-like* gene expression patterns, and possibly extensive redundancy with other transcription factors in places where functions have not been detected.

In contrast, FUL-like proteins vary in their ability to interact with floral organ identity proteins. For instance, whereas *Euptelea* (EuplFL1 and EuplFL2) and *Aquilegia* (AqFL1) FUL-like proteins only interact with SEPALLATA proteins (Liu et al., 2010) (L. Holappa & E. Kramer pers. comm.), *Akebia* (Lardizabalaceae) FUL-like proteins can interact with SEPALLATA , PISTILLATA and AGAMOUS orthologs (Liu et al., 2010). Likewise, interaction between FUL-like proteins and floral organ identity proteins has been detected in *Lolium* and *Oryza* (Moon et al., 1999; Fornara et al., 2004; Ciannamea et al., 2006). Unfortunately, there are no comparable data for any Papaveraceae, and data on the interactions of MADS-domain proteins outside of the core-eudicots is limited to few species. Nonetheless the data available do suggest that in different taxa FUL-like protein interactions vary significantly, suggesting in turn a potential source for functional variation attributed to *FUL-like* genes.

5.3.d. Character evolution in Ranunculales and the function of *FUL-like* genes

Plants in the order Ranunculales vary greatly in terms of habitat and floral diversity (RonseDeCraene et al., 2003; Soltis et al., 2003; Soltis et al., 2005). In the last decade a some functional data regarding the molecular basis of floral development in a few taxa in the Ranunculales has increased and thus, a study of character evolution linked with data on gene expression and function allows us to make direct comparisons with data collected from core eudicots (Drea et al., 2007; Kramer et al., 2007; DiStilio et al., 2010; Yellina et al., 2010; Dreni et al., 2011; Hands et al., 2011; Sharma et al., 2011). However, the number of species sampled is still vey restricted to evaluate functional trends across the order. The available functional data suggests that *FUL-like* genes are important in the sculpting of inflorescence structure across Ranunculales, as they control the elongation of axillary buds in cauline leaves and rosette leaves in the three species for which there is functional data (*Eschscholzia californica*, *Papaver somniferum* and *Aquilegia coerulea*). This role seems to be conserved in early diverging families like Papaveraceae as well as in the derived genus *Aquilegia*. Evolution of other functions is more complicated. For instance, the roles of *FUL-like* genes in floral meristem identity, perianth identity, and fruit development appear to have been acquired before the diversification of the Papaveraceae and lost again at some point in the evolution of Ranunculiids before the diversification of *Aquilegia*. Alternatively, these roles are unique to Papaveracaeae as they are the only members of Ranunculales with sepaloid sepals and a syncarpic gynoecium, and *FUL-like* genes play different roles in taxa with petaloid sepals and apocarpic gynoecium, including the majority of Lardizabalaceae, Menispermaceae, Berberidaceae and Ranunculaceae. At this point is also unclear when *FUL-like* genes acquired a role in leaf complexity and shape. They do not play a role in the simple leaved *Papaver*, and they don’t seem to play a role in the compound leaved *Eschscholzia* (although only two out of three paralogs were silenced in previous experiments) suggesting that *FUL-like* genes acquired the role in leaf morphogenesis after the evolution of Papaveraceae at any point before the evolution of Aquilegia. On the whole the data highlights the fact that more functional data in species with specific character combinations across the order, particularly those between Papaveraceae and Ranunculaceae, are needed to understand *FUL-like* gene functional evolution, at a more local scale, during the evolution of the Ranunculales.

5.4. MATERIALS AND METHODS

5.4.a. Character Optimization

A composite tree was generated based on published trees (Kadereit et al., 1994, 1995; Hoot et al., 1997; Hoot et al., 1999; Wang et al., 2009) for the Ranunculales, including a sampling parallel to the one used for *FUL-like* genes. Seven morphological characters correlated with functions attributed to *FUL-like* genes (Chapter 3) were optimized on this phylogeny and were coded as follows: ***1. Leaf type:*** (0) simple, (1) compound (2) variable within the genus; ***2. Leaf margin****:* (0) entire, (1) serrate, (2) lobed (3) variable within the genus; **3. *Inflorescence determinacy****:* (0) cyme, (1) raceme; **4. *Inflorescence branching orders:*** (0) terminal flower only – no branching, (1) one order of branches, (2) two orders of branches, (3) more than 2 orders of branches; **5. *Sepal morphology***: (0) sepaloid, (1) petaloid, (2) absent; ***6. Sepal persistence****:* (0) persistent , (1) caducous, (2) absent; **7. *Sepal fusion degree****:* (0) free sepals, (1) fused sepals, (2) absent; ***8. Gynoecium type*** : (0) syncarpous, (1) apocarpous, (2) unicarpellate. **9. *Fruit type****:* (0) dry dehiscent fruit (capsule / follicle), (1) fleshy indehiscent fruit (berry), (2) dry indehiscent fruit (achene). Character states were compiled from a number of sources: Günther, 1975; Kadereit, 1993; Kadereit et al., 1994; Loconte et al., 1995; Nickol, 1995; Schwarzbach and Kadereit, 1995; Takhtajan, 1997; Wang et al., 2009, http://www.efloras.org/ and herbarium specimens deposited at NYBG. Ancestral character states were reconstructed in MacClade 4.08 using parsimony with equal cost of state changes to reconstruct all states at each node (Maddison and Maddison, 2000).

5.4.b. Plant Material

Leaf and floral tissue was obtained from a number of basal eudicots, mostly within Papaveraceae s.l. and Ranunculaceae, as well as non-eudicots mostly within Piperales. Species were selected on the basis of availability of leaf or floral material. Fersh material was obtained from living collections at The New York Botanical Garden or at the Systematics Garden at Lehman College. Voucher information for all of these species is in Table 1.

5.4.c. Cloning and characterization of *FUL-like* genes

Total RNA was extracted from 0.5-1 g of young leaf or floral buds using TRIZOL reagent (Invitrogen) and was DNaseI-treated (Roche) to remove residual genomic DNA. 2µg were used as template for cDNA synthesis with SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The resulting cDNA was diluted 1:10. Initial amplifications using degenerate primers to recover a pool of MADS-box genes were done as in Litt and Irish (2003), with two modifications; 1) the amplification program began with a 5 min activation step at 95°C, and five initial cycles with an incubation step of 30 sec at 95°C, a 30 sec annealing step at 42°C and a 1 min extension at 72°C, followed by 30 cycles with an incubation step at 95°C for 30 sec, a 30 sec annealing step at 50°C and a 1 min extension at 72°C. The products of this amplification were diluted 1:20 and used as templates in successive reactions. These reactions were done as in Litt and Irish (2003) with the same amplification program. In addition to the primers used by Litt and Irish (2003) the forward degenerate primer ATGGRDAGAGGWAGGGTWCAG, designed to bind the beginning of the MADS domain, was used in combination with all degenerate reverse primers designed to amplify the full coding sequence, including the complete 5'end, of the *FUL-like* genes. All PCR products were run on a 1% agarose gel and amplicons between 600-900 bp in size were cloned into pCR®2.1-TOPO® (Invitrogen).

In addition, 23 previously published sequences of *FUL-like* genes from basal angiosperms, basal eudicots and grasses (Litt and Irish, 2003; Preston and Kellogg, 2006; Shan et al., 2007) were retrieved from GenBank using BLAST (Altschul et al., 1990) and 8 new unpublished *FUL-like* sequences were retrieved from www.phytometasyn.ca.

5.4.d. Phylogenetic Analyses

Between 40 and 60 clones were sequenced per species. If variation was found among clones, the criteria to distinguish allelic variation at a single locus from different loci was the same used by Litt and Irish (2003). Sequences were compiled using Bioedit and then aligned using the online version of MAFFT (Katoh et al., 2002) at http://mafft.cbrc.jp/alignment/server/, with a gap open penalty of 3.0, an offset value of 0.2, and all other default settings. The alignment was then refined by hand using Bioedit and MacClade 4.06 (Maddison and Maddison, 2000). Two different versions were saved, one with nucleotides and the other with amino acids (AA). The nucleotide alignment for full-length sequences was used for phylogenetic analyses. The AA alignment was used to identify conserved AA motifs as well as diagnostic single AAs and; these were mapped on the gene tree generated. Not surprisingly the highly conserved MADS domain was very easy to align whereas the variable C-portion of the protein was more difficult to align. Indels in this region are due to a combination of expansion in repetitive sequences. The magnoliid sequences (Ma.gr.AP1 and Pe.am.AP1) were used to root the trees, and all non-Ranunculales sequences were used as outgroup. These include basal angiosperms from the Piperales , Magnoliales, Laurales and Grasses.

Parsimony trees were generated using TNTv1.1 (Goloboff et al., 2008). Heuristic searches were performed with 2000 parsimony ratchet replicates (500 iteration ratchet), holding 20 trees per ratchet, followed by tree-bisection-reconnection (TBR)-max branch swapping. Support for nodes was calculated by bootstrap analyses (BS) with 1000 replicates doing 10 ratchets per replicate, collapsing nodes that had under 40% bootstrap support. Maximum Likelihood (ML) phylogenetic analyses were performed in RaxML-HPC2 BlackBox (Stamatakis et al., 2005) on the Cipres Science Gateway (Miller et al., 2009). The Best performing evolutionary model was obtained by the Akaike information criterion (AIC) using the program jModelTest v.0.1.1 (Posada and Crandall, 1998). Bootstrapping was performed according to the default criteria in RAxML: Bootstrapping stopped after 200 replicates when the criteria were met.

5.4.e. Relative rates of evolution

The ML highest likelihood tree with branch lengths and bootstrap replicates was analyzed using r8s (Sanderson, 2002) to search for relative rates of evolution at selected nodes. The likelihood ratio-based relative test (rrlike) was implemented to infer local shifts in rates of evolution in selected clades. No nodes were constrained and age was arbitrarily set at 1.0 for all the clades. Likelihood ratios of clock vs. non clock evolution rates were analyzed for each node for each bootstrap replicate tree.

Table 5.1. Accession numbers of *FUL-like* sequences used in this study.

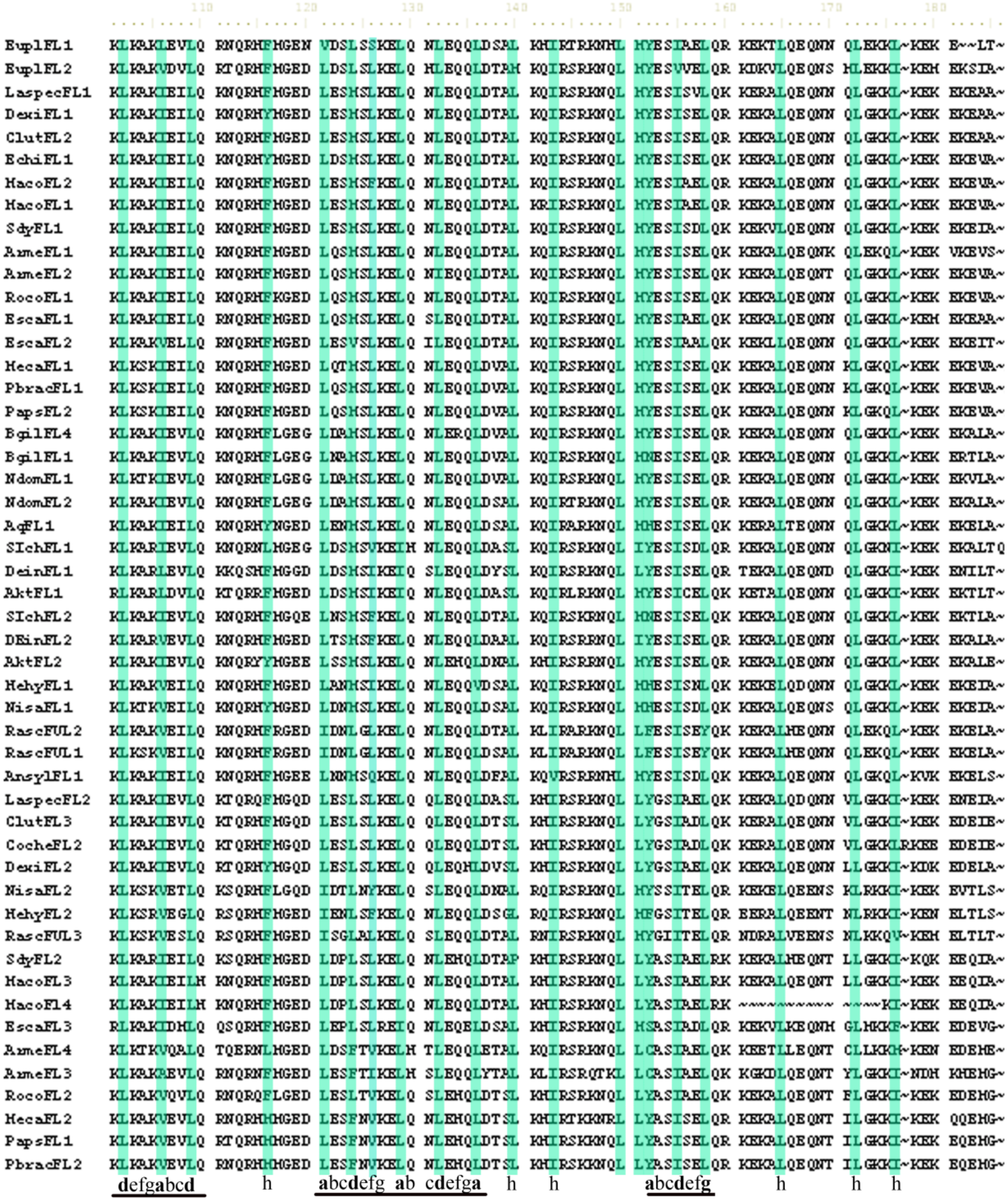
In **bold** Sequences identified in this work; * Sequences retrieved from BLAST; ~ sequences identified through www.phytometasyn.ca. Source material for FUL-like gene amplification at the laboratory comes from the living collections at the New York Botanical Garden in the Rock Garden, or at the Systematics Garden at Lehman College.

Gene name	**Species name**	**Family - Order**	**Accession number**
Outgroup			
*MagrAP1**	*Magnoliagrandiflora*	Magnoliaceae - Magnoliales	
*PeamAP1**	*Persea Americana*	Lauraceae - Laurales?	
AcauFL1	*Asarum caudatum*	Aristolochiaceae - Piperales	
AeurFL1	*Asarum europaeum*		
AeurFL2			
AeurFL3			
ShenFL1	*Saruma henryi*		
AtriFL1	*Aristolochia trilobata*		
AtriFL2			
AfimFL1	*Aristolochia fimbriata*		
AsalFL1	*Aristolochia salvadorensis*		
AsalFL2			
AsalFL3			
AsalFL4			
*HcAP1**	*Houttuynia cordata*	- Piperales	
Ingroup			
*EuplFL1**	*Euptelea pleiosperma*	Eupteleaceae - Ranunculales	
*EuplFL2**			
LaspecFL1	*Lamprocapnos spectabilis*	Fumariaceae - Ranunculales	
LaspecFL2			
DexiFL1	*Dicentra eximia*		
DexiFL2			
CocheFL1	*Corydalis cheilantifolia*		
CocheFL2			
ClutFL1	*Corydalis lutea*		
ClutFL2			
DascaFL1	*Dactylicapnos scandens*		
DascaFL2			
CaseFL1	*Capnoides sempervirens*		
CyveFL1	*Cisticapnos vesicarius*		
EchiFL1	*Eomecon chionantha*	Papaveraceae - Ranunculales	
MacoFL1	*Macleaya cordata*		
MacoFL2			
MacoFL3			
MacoFL4			

BofrFL1	*Bocconia frutescens*		
BofrFL2			
*CmFL1**	*Chelidonium majus*		
*CmFL2**			
SdyFL1	*Stylophorum diphyllum*		
SdyFL2			
EscaFL1	*Eschscholzia californica*		
EscaFL2			
EscaFL3 ~			
ArmeFL1 ~	*Argemone Mexicana*		
ArmeFL2 ~			
ArmeFL3 ~			
ArmeFL4 ~			
RocoFL1	*Romneya coulteri*		
RocoFL2			
MecaFL1	*Meconopsis cambrica*		
MecaFL2			
PapsFL1	*Papaver somniferum*		
PapsFL2			
*PapnFL1**	*Papaver nudicaule*		
PrhFL1	*Papaver rhoeas*		
PrhFL2			
PbracFL1~	*Papaver bracteatum*		
PbracFL2~			
PatlaFL	*Papaver atlanticum*		
*SIchFL1**	*Sinofranchetia chinensis*	Lardizabalaceae - Ranunculales	
*SIchFL2**			
*DEinFL1**	*Decaisnea insignis*		
*DEinFL2**			
*AktFL1**	*Akebia trifoliate*		
*AktFL2**			
NisaFL1 ~	*Nigella sativa*	Ranunculaceae - Ranunculales	
NisaFL2 ~			
HehyFL1	*Helleborus hybrida*		
HehyFL2			
AqFL1	*Aquilegia coerulea*		
AnsylFL1	*Anemone sylvestris*		
ErhyeFL1	*Eranthis hyemalis*		
ErhyeFL2			
*RascFUL1**	*Ranunculus sceleratus*		
*RascFUL2**			
*RascFUL3**			
*RbFL1**	*Ranunculus bulbosus*		
*RbFL2**			
*RbFL3**			
*RbFL4**			
ClejoeFL1	*Clematis sp. ‘cv joe’*		

5.5. APPENDIX

5.5.a. Supplemental Figure 1. K-domain sequence alignment of Ranunculiid FUL-like proteins. Hydrophobic amino-acids in **a** and **d** positions in the heptad (**a**bc**d**efg)$_n$ repeats are in bold. The predicted protein sequence at this domain contains three amphipathic α-helices: K1, K2 and K3. Conserved hydrophobic amino-acids outside of the predicted helices are highlighted and labeled with **h**.



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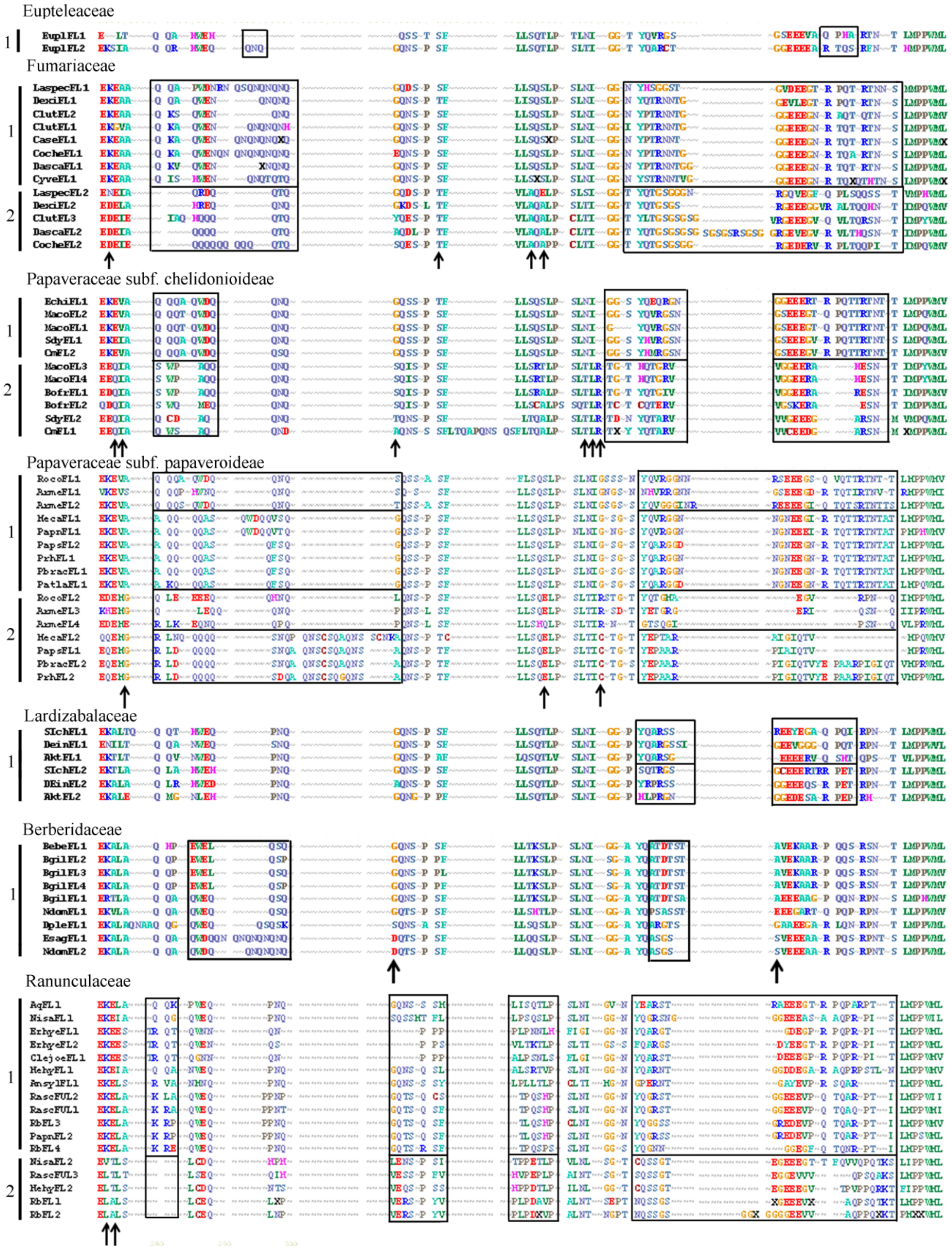
                    110        120        130        140        150        160        170        180
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|.
EuplFL1     KLKAKLEVLQ RNQRHFHGEN VDSLSSKELQ NLEQQLDSAL KHIRTRKNHL HYESIAELQR KEKTLQEQNN QLEKKL~KEK E~~LT~
EuplFL2     KLKAKVDVLQ KTQRHFHGED LDSLSLKELQ HLEQQLDTAH KQIRSRKNQL HYESVVELQR KDKVLQEQNS HLEKKI~KEH EKSIA~
LaspecFL1   KLKAKIEILQ KNQRHFHGED LESHSLKELQ NLEQQLDTAL KQIRSRKNQL HYESISVLQK KERALQEQNN QLGKKL~KEK EKEAA~
DexiFL1     KLKAKIEILQ KNQRHYHGED LESHSLKELQ NLEQQLDTAL KQIRSRKNQL HYESISELQR KEKALQEQNN QLGKKL~KEK EKEAA~
ClutFL2     KLKAKIEILQ KNQRHFHGED LESHSLKELQ NLEQQLDTAL KQIRSRKNQL HYESISELQR KEKALQEQNN QLGKKL~KEK EKEAA~
EchiFL1     KLKAKIEILQ KNQRHYHGED LDSHSLKELQ NLEQQLDTAL KQIRSRKNQL HYESISELQK KEKALQEQNN QLGKKL~KEK EKEVA~
HacoFL2     KLKAKIEILQ KNQRHFHGED LESHSFKELQ NLEQQLDTAL KQIRSRKNQL HYESIAELQR KEKALQEQNN QLGKKL~KEK EKEVA~
HacoFL1     KLKAKIEILQ KNQRHFHGED LESHSLKELQ NLEQQLDTAL KRIRSRKNQL HYESIAELQR KEKALQEQNN QLGKKL~KEK EKEVA~
SdyFL1      KLKAKIEILQ KNQRHFHGED LESHSLKELQ NLEQQLDTAL KQIRSRKNQL HYESISDLQK KEKVLQEQNN QLGKKL~KEK EKEIA~
AxmeFL1     KLKAKIEILQ KNQRHFHGED LQSHSLKELQ NLEQQLDTAL KQIRSRKNQL HYESISELQK KEKALQEQNK QLEKQL~KEK VKEVS~
AxmeFL2     KLKAKIEILQ KNQRHFHGED LQSHSLKELQ NIEQQLDTAL KQIRSRKNQL HYESISELQK KEKALQEQNT QLGKKL~KEK EKEVA~
RocoFL1     KLKAKIEILQ KNQRHFKGED LQSHSLKELQ NLEQQLDTAL KQIRSRKNQL HYESISELQK KEKALQEQNN QLGKKL~KEK EKEVA~
EscaFL1     KLKAKIEILQ RNQRHFHGED LQSHSLKELQ SLEQQLDTAL KQIRSRKNQL HYESIAELQK KEKALQEQNN QLGKKL~KEH EKEAA~
EscaFL2     KLKAKVELLQ RNQRHFHGED LESVSLKELQ ILEQQLDTAL KQIRSRKNQL HYESIAALQK KEKLLQEQNN QLGKKL~KEK EKEIT~
HecaFL1     KLKSKIEILQ KNQRHFHGED LQTHSLKELQ NLEQQLDVAL KQIRSRKNQL HYESISELQK KEKALQEQNN KLGKQL~KEK EKEVA~
PbracFL1    KLKSKIEILQ KNQRHFHGED LQSHSLKELQ NLEQQLDVAL KQIRSRKNQL HYESISELQK KEKALQEQNN KLGKQL~KEK EKEVA~
PapsFL2     KLKSKIEILQ KNQRHFHGED LQSHSLKELQ NLEQQLDVAL KQIRSRKNQL HYESISELQK KEKALQEQNN KLGKQL~KEK EKEVA~
BgilFL4     KLKAKIEVLQ KNQRHFLGEG LDAHSLKELQ NLERQLDVAL KQIRSRKNQL HYESISELQR KEKALQEQNN QLGKKL~KEK EKALA~
BgilFL1     KLKAKIEVLQ KNQRHFLGEG LNAHSLKELQ NLEQQLDVAL KQIRSRKNQL HNESISELQR KEKALQEQNN QLGKKL~KEK ERTLA~
NdomFL1     KLKTKIEVLQ KNQRHFLGEG LDAHSLKELQ NLEQQLDVAL KQIRSRKNQL HYESISELQR KEKALQEQNN QLGKKL~KEK EKVLA~
NdomFL2     KLKAKIEVLQ KNQRHFLGEG LDAHSLKELQ NLEQQLDSAL KQIRTRKNQL HYESISELQR KEKALQEQNN QLGKKL~KEK EKALA~
AqFL1       KLKAKIEILQ KNQRHYNGED LENHSLKELQ NLEQQLDSAL KQIRARKNQL HNESISELQK KERALTEQNN QLGKKL~KEK EKELA~
SIchFL1     KLKARIEVLQ KNQRNLHGEG LDSHSVKEIH NLEQQLDASL KQIRSRKNQL IYESISDLQR KEKALQEQNN QLGKNI~KEK EKALTQ
DeinFL1     KLKARLEVLQ KKQSHFHGGD LDSHSIKEIQ SLEQQLDYSL KQIRSRKNQL LYESISELQR TEKALQEQND QLGKKI~KEK ENILT~
AktFL1      RLKARLDVLQ KTQRRFHGED LDSHSIKEIQ NLEQQLDASL KQIRLRKNQL HYESICELQK KETALQEQNN QLGKKI~KEK EKTLT~
SIchFL2     KLKAKIEVLQ KNQRHFHGQE LNSHSFKELQ NLEQQLDTAL KQIRSKRNQL HNESISELQK KEKALQEQNN QLGKKL~KEK EKTLA~
DEinFL2     KLKARVEVLQ KNQRHFHGED LTSHSFKELQ NLEQQLDAAL KQIRSRRNQL IYESISELQR KEKALQEQNN QLGKKL~KEK EKALA~
AktFL2      KLKAKIEVLQ KNQRYYHGEE LSSHSLKELQ NLEHQLDNAL KHIRSRRNQL HYESISELQR KEKALQEQNN QLGKKL~KEK EKALE~
HehyFL1     KLKAKVEILQ KNQRHFHGED LANHSIKELQ NLEQQVDSAL KQIRSRKNQL HNESISNLQK KEKELQDQNN QLGKKL~KEK EKEIA~
NisaFL1     KLKTKVEILQ KNQRHYHGED LDNHSLKELQ NLEQQLDSAL KQIRSRKNQL HNESISDLQK KEKALQEQNS QLGKKL~KEK EKEIA~
RascFUL2    KLKAKVEILQ KNQRHFRGED IDNLGLKELQ NLEQQLDTAL KLIRARKNQL LFESISEYQK KEKALHEQNN QLEKQL~KEK EKELA~
RascFUL1    KLKSKVEILQ KNQRHFRGED IDNLGLKELQ NLEQQLDSAL KLIRARKNQL LFESISEYQK KEKALHEQNN QLEKQL~KEK EKELA~
AnsylFL1    KLKAKIEILQ KNQRHFHGEE LNNHSQKELQ NLEQQLDFAL KQVRSRRNHL HYESISDLQK KEKALQEQNN QLGKQL~KVK EKELS~
LaspecFL2   KLKAKIEVLQ KTQRQFHGQD LESLSLKELQ QLEQQLDASL KHIRSRKNQL LYGSIAELQK KEKALQDQNN VLGKKI~KEK ENEIA~
ClutFL3     KLKAKIEVLQ KTQRHFHGQD LESLSLKELQ QLEQQLDTSL KHIRSRKNQL LYGSIADLQK KERALQEQNN VLGKKI~KEK EDEIE~
CocheFL2    KLKAKIEVLQ KTQRHFHGQD LESLSLKELQ QLEQQLDTSL KHIRSRKNQL LYGSIADLQK KERALQEQNN VLGKKLRKEE EDEIE~
DexiFL2     KLKAKIEVLQ KTQRHYHGQD LESLSLKELQ QLEQHLDVSL KHIRSRKNQL LYGSIAELQK KEKALQEQNN LLGKKI~KDK EDELA~
NisaFL2     KLKSKVETLQ KSQRHFLGQD IDTLNYKELQ SLEQQLDNAL RQIRSRKNQL HYSSITELQR KEKELQEENS KLRKKI~KEK EVTLS~
HehyFL2     KLKSRVEGLQ RSQRHFHGED IENLSFKELQ NLEQQLDSGL RQIRSRKNQL HFGSITELQR EERALQEENT NLRKKI~KEN ELTLS~
RascFUL3    KLKSKVESLQ RSQRHFHGED ISGLALKELQ SLEQQLDTAL RNIRSRKNQL HYGIITELQR NDRALVEENS NLKKQV~KEH ELTLT~
SdyFL2      KLKARIEILQ KSQRHFHGED LDPLSLKELQ NLEHQLDTAP KHIRSRKNQL LYASIAELRK KEKALHEQNT LLGKKI~KQK EEQIA~
HacoFL3     KLKAKIEILH KNQRHFHGED LDPLSLKELQ NLEQQLDTAL KHIRSRKNQL LYASIAELRK KEKALQEQNT LLGKKI~KEK EEQIA~
HacoFl4     KLKAKIEILH KNQRHFHGED LDPLSLKELQ NLEQQLDTAL KHIRSRKNQL LYASIAELRK ~~~~~~~~~~ ~~~~KI~KEK EEQIA~
EscaFL3     RLKAKIDHLQ QSQRHFHGED LEPLSLREIQ NLEQELDSAL KHIRSRKNQL HSASIADLQR KEKVLKEQNH GLHKKF~KEK EDEVG~
AxmeFL4     KLKTKVQALQ TQERNLHGED LDSFTVKELH TLEQQLETAL KQIRSRKNQL LCASIAELQK KEETLLEQNT CLLKKH~KEN EDEHE~
AxmeFL3     KLKAKAEVLQ RNQRNFHGED LESFTIKELH SLEQQLYTAL KLIRSRQTKL LCASIAELQK KGKDLQEQNT YLGKKI~NDH KHEHG~
RocoFL2     KLKAKVQVLQ RNQRQFLGED LESLTVKELQ SLEHQLDTSL KHIRSRKNQL LYASIAELQK KEKALQEQNT FLGKKI~KEK EDEHG~
HecaFL2     KLKAKVEVLQ RNQRHHHGED LESFNVKELQ NLEHQLDTSL KHIKTKKNRL LYASISELQR KEKALQEQNT ILGKKI~KEK QQEHG~
PapsFL1     KLKAKVEVLQ KTQRHHHGED LESFNVKELQ NLEHQLDTSL KHIRSKKNQL LYASISELQR KEKALQEQNT ILGKKI~KEK EQEHG~
PbracFL2    KLKAKVEVLQ RNQRHHHGED LESFNVKELQ NLEHQLDTSL KHIRSKKNQL LYASISELQR KEKALQEQNT ILGKKI~KEK EQEHG~
```

5.5.b. Supplemental Figure 2. Alignment of the end of the K domain and the C-terminal domain organized by families and paralogous clades (1 and 2). Boxes represent the regions that are variable among paralogs but relatively conserved in orthologs in each family or subfamily (in Papaveraceae).



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