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RESEARCH ARTICLE

Divergent Functional Diversification Patterns in the SEP/AGL6/AP1 MADS-box Transcription Factor Superclade

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Short title: Analysis of the Petunia SEP/AGL6/AP1 Superclade

One sentence summary: Functional analysis of the petunia MADS-box gene SEP/AGL6/AP1 superclade compared to Arabidopsis and other species suggests major differences in the functional diversification of its members during evolution.

Keywords: SEPALATA; APETALA1; AP1/SQUA; AGL6; MADS-box; floral meristem identity; inflorescence meristem identity, plant evolution; ABC model; Petunia; Arabidopsis; inflorescence architecture

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ABSTRACT

Members of SEPALATA (SEP) and APETALA1 (AP1)/SQUAMOSA (SQUA) MADS-box transcription factor subfamilies play key roles in floral organ identity determination and floral meristem determinacy in the Rosid species Arabidopsis. Here, we present a functional characterization of the seven SEP/AGL6 and four AP1/SQUA genes in the distant Asterid species Petunia x hybrida petunia. Based on the analysis of single and higher order mutants, we report that the petunia SEP1/SEP2/SEP3 orthologs together with AGL6 encode classical SEP floral organ identity and floral termination functions, with a master role for the petunia SEP3 ortholog FLORAL BINDING PROTEIN 2 (FBP2). By contrast, the FBP9 subclade members FBP9 and FBP23, for which no clear ortholog is present in Arabidopsis, play a major role in determining floral meristem identity together with FBP4, while contributing only moderately to floral organ identity. In turn, the four members of the petunia AP1/SQUA subfamily redundantly are required for inflorescence meristem identity, and act as B-function repressors in the first floral whorl, together with BEN/ROB genes. Overall, these data together with studies in other species suggest major differences in the functional diversification of the SEP/AGL6 and AP1/SQUA MADS-box subfamilies during angiosperm evolution.
INTRODUCTION

Over the last two decades, the ABC model of floral organ identity has served as a genetic framework for the understanding of flower development in other species, and across evolution (Bowman et al., 2012). Members of the MADS-box transcription factor family play a central role in this model, and especially the MADS-BOX proteins encoding the floral B- and C-functions have been studied in a wide range of species (Krizek and Fletcher, 2005), providing a better understanding of the evolution and diversification of floral development at the molecular level. By contrast, much less comparative data is available for members of the AP1/SQUA and the SEPALLATA MADS-box transcription factor subfamilies. Compared to the B- and C-class MADS-box subfamilies, the SEP and AP1/SQUA subfamilies have substantially expanded via several gene duplication events during angiosperm evolution (Litt and Irish, 2003; Zahn et al., 2005). Together with reported extensive redundancy among individual SEP and among AP1/SQUA genes (see below), this makes comparative functional studies challenging, and probably underlies the relative lack of functional data in a broad range of species. Moreover, the extensive sequence similarity observed among members within both subfamilies may render the interpretation of phenotypes obtained by gene-silencing approaches (such as RNAi/co-suppression/VIGS) difficult. In addition, in several species members of the closely related AGL6 MADS-box subfamily also perform SEP-like functions (Ohmori et al., 2009; Rijpkema et al., 2009; Thompson et al., 2009; Dreni and Zhang, 2016), adding further genetic complexity to a comparative analysis of the SEP function across species borders.

The SEP and AP1/SQUA MADS-box transcription factor families are unique to angiosperms, while AGL6 genes are present both in gymnosperms and angiosperms (Becker and Theissen, 2003; Litt and Irish, 2003; Zahn et al., 2005). Interestingly, the AGL6, SEP and AP1/SQUA subfamilies together compose a monophyletic superclade within the MADS-box family (further referred to as the AP1/SEP/AGL6 superclade), suggesting a common ancestral origin predating the angiosperm/gymnosperm divergence, although the evolutionary relationship between the different subfamilies had not been completely resolved (Purugganan et al., 1995; Purugganan, 1997; Becker and Theissen, 2003). A more recent phylogenetic analysis based on exon/intron structural changes suggests that AGL6 genes are sister to both SEP and API subfamilies (Yu et al., 2016).

Thus far, Arabidopsis is the only core eudicot species for which a functional characterization of all its AP1/SEP/AGL6 superclade genes has been achieved in sufficient detail, including the identification of redundant functions through higher order mutant analysis, but a wealth of functional data has been accumulated also in tomato and rice in recent years.
The Arabidopsis SEP subfamily consists of four members, named SEP1, SEP2, SEP3 and SEP4, and petals, stamens and carpels in the sep1 sep2 sep3 triple mutant are transformed into sepals (Pelaz et al., 2000), while all floral organs in a sep1 sep2 sep3 sep4 mutant develop as leaf-like organs (Ditta et al., 2004). This led to the conclusion that SEP genes are required for the identity of all floral organs, and function in a largely, but not completely redundant fashion. In addition, SEP genes were shown to be involved in floral meristem identity and determinacy (Pelaz et al., 2000; Ditta et al., 2004). SEP proteins are proposed to act as ‘bridge proteins’ enabling higher order complex formation (floral quartets) with the products of the homeotic B and C function organ identity genes, and to provide transcriptional activation capacity to these complexes (Honma and Goto, 2001; Theissen and Saedler, 2001; Immink et al., 2009; Melzer et al., 2009). These findings have inspired the addition of the SEP (or E-) function to the classic ABC model of floral development (Bowman et al., 1991; Coen and Meyerowitz, 1991), summarized in a floral quartet model (Theissen and Saedler, 2001). In contrast to the function of AGL6 genes in other species, the two Arabidopsis AGL6 subfamily members AGL6 and AGL13 do not seem to perform a SEP-like function in floral organ identity determination (Koo et al., 2010; Huang et al., 2012; Hsu et al., 2014). The Arabidopsis AP1/SQUA subfamily is composed of 4 members, of which the roles of AP1, CAL (CAULIFLOWER) and FUL (FRUITFULL) in floral development have been particularly well studied. Arabidopsis ap1 mutants lack petals and have sepals displaying bract like features (Irish and Sussex, 1990; Mandel et al., 1992). For these reasons, AP1 has been classified as an A-function gene in the ABC model, required for the identity specification of sepals and petals. Furthermore, AP1 plays also a major role in specifying floral meristem identity, in a largely redundant fashion with CAL (Bowman et al., 1993; Kempin et al., 1995). FUL was initially identified for its unique role in Arabidopsis carpel and fruit development (Gu et al., 1998), but in addition was later shown to function redundantly with AP1 and CAL to control inflorescence architecture (Ferrandiz et al., 2000).

To provide more insight in floral development and in the evolution of the floral gene regulatory network in higher eudicot species in general, we have been systematically analyzing the genetics underlying floral development in the Asterid species Petunia x hybrida. While the genes encoding the floral A, B and C- functions in petunia have been well characterized (Angenent et al., 1993; van der Krol et al., 1993; Kater et al., 1998; Kapoor et al., 2002; Vandenbussche et al., 2004; Rijpkema et al., 2006; Cartolano et al., 2007; Heijmans et al., 2012; Morel et al., 2017; Morel et al., 2018), only a few of the 10 previously described genes belonging to the large petunia AP1/SEP/AGL6 superclade (Immink et al., 1999; Ferrario et al.,
Research on petunia *SEPALLATA* genes dates back a long time and provided, together with a study in tomato, the first indication of the existence of SEP-function in floral development: transgenic lines in which the *SEP3*-like petunia *FBP2* or tomato *TM5* genes were silenced by co-suppression both exhibited simultaneous homeotic conversion of whorls 2, 3, and 4 into sepal-like organs and loss of determinacy in the center of the flower (Angenent et al., 1994; Pnueli et al., 1994), a phenotype similar to that later found in Arabidopsis *sep1 sep2 sep3* mutants. However, at that time, multimeric complex formation of MADS-box proteins still remained to be discovered (Egea-Cortines et al., 1999), and it was not clear how many genes where co-suppressed in these lines. Therefore, the molecular basis of these phenotypes in petunia and tomato was not immediately understood. Later, it was shown in yeast that petunia SEP proteins also bind to B-class heterodimers and to C-class proteins, mediate quaternary complex formation with B- and C-class proteins and display transcriptional activation capacity (Ferrario et al., 2003), compatible with the proposed quartet model in Arabidopsis. Interestingly, among the six petunia SEP-like proteins, also clear differences in protein–protein interactions were revealed in a yeast 2-hybrid assay, suggesting functional diversification (Ferrario et al., 2003; Immink et al., 2003). Especially *FBP2* and *FBP5* showed a much broader range of interaction partners compared to the other petunia SEP proteins. Furthermore, it was shown *in planta* that petunia SEP proteins may be crucial to import at least some other MADS-box transcription factors into the nucleus (Immink et al., 2002).

Using a gene-specific approach, we showed that the *fbp2* co-suppression phenotype was indeed not gene specific, since single *fbp2* mutants showed only a very incomplete *sep-like* phenotype, with primarily the margins of the petals exhibiting a petal-to-sepal homeotic conversion (Vandenbussche et al., 2003b). We also reported *fbp5* mutants that as single mutants develop as wild type. Flowers of *fbp2 fbp5* mutants, however, showed an enhanced phenotype compared to *fbp2* mutants: the sepaloid regions at the petal edges extended slightly further towards the center; sepal-like structures appeared on top of the anthers, and a sudden dramatic phenotype appeared in the ovary, which continued to grow long after development has arrested in wild-type (WT) flowers of comparable stages, resulting in a giant ovary. While the general architecture of the ovary was maintained (carpels containing an interior placenta), inside all ovules were homeotically converted to sepal-like organs (Vandenbussche et al., 2003b). This directly demonstrated that not only the identity of petals, stamens and carpels depends on SEP activity in petunia, but also ovule identity, as was also reported in Arabidopsis in the same
journal issue (Favaro et al., 2003). More recently, we demonstrated that petunia AGL6 also exhibits SEP-like functions (Rijpkema et al., 2009), and performs a major role in petal identity, redundantly with FBP2. In addition, a function in stamen development was revealed by fbp5/afb9, fbp5 agl6 triple mutant analysis. In line with the proposed SEP-function for AGL6, we found that AGL6 and FBP2 in yeast overall interact with the same the partners (Rijpkema et al., 2009).

Thus far, three petunia AP1/SQUA genes have been described, called PFG, FBP26 and FBP29 (Immink et al., 2003), and only the function of PFG was analyzed, using a co-suppression approach, resulting in a dramatic nonflowering phenotype, although the occasional development of single solitary flowers in these lines was also reported (Immink et al., 1999). However, as for the FBP2 co-suppression line, the full-length coding sequence including the highly conserved MADS-domain was used to generate the co-suppression construct, questioning the specificity of the obtained phenotype.

To provide more insight in the functions of the AP1/SEP/AGL6 superclade members in petunia, and more broadly in the evolutionary trajectory of the AP1/SEP/AGL6 superclade in the core eudicots, we aimed to uncover unique and redundant functions of the complete SEP/AGL6 and AP1/SQUA subfamilies during petunia flower development.

First, we present a genetic fine-dissection of the petunia SEP-function obtained from the analysis of a series of single and multiple knock-out mutants, combining putative null mutations in the six petunia SEP genes and AGL6. Most remarkably, we found that the FBP9 subclade members FBP9 and FBP23, for which no clear ortholog is present in Arabidopsis (Zahn et al., 2005), play an essential role in determining floral meristem identity together with FBP4, with only moderate contributions to the classic SEP floral organ identity function. Furthermore, we show that the petunia genetic equivalent of the Arabidopsis sep1 sep2 sep3 mutant still displays residual B- and C-function activity, while a full sepallata phenotype was obtained in a sextuple fbp2 fbp4 fbp5 fbp9 pm12 agl6 mutant. The analysis further suggests that the petunia SEP3 ortholog FBP2 performs a master floral organ identity SEP-function as in Arabidopsis. In addition, we have analyzed the dependence of homeotic gene expression on the SEP function, by comparing the dynamics of expression between wild-type and the sextuple fbp2 fbp4 fbp5 fbp9 pm12 agl6 mutant.

Finally, we show that the petunia AP1/SQUA subfamily is composed of four members (PFG, FBP26, FBP26 and the here described euAP1 gene) that function in a largely redundant way. We found that they are required for inflorescence meristem identity, but surprisingly, pfg fbp26 fbp29 euap1 mutants developed fully functional terminal flowers. In addition, we show that they act as B-function repressors in the first floral whorl, together with BEN/ROB genes
(Morel et al., 2017). Overall, comparison of these data with previous studies in mainly Arabidopsis, tomato and rice reveal major differences in the functional diversification of the SEP/AGL6 and AP1/SQUA MADS-box subfamilies during evolution of the angiosperms.

RESULTS

Petunia Floral Development

To facilitate the comparison of the phenotypes presented in this study with the equivalent Arabidopsis mutants, we summarize first the relevant differences in WT floral architecture between petunia and Arabidopsis (Figure 1). Petunia flowers consist, from the outside towards the center, of five sepals partly fused at their basis, five large congenitally fused petals, five stamens of which the filaments are partly fused with the petal tube, and a central pistil composed of two congenitally fused carpels (Figure 1A). Some important differences in flower development between petunia and Arabidopsis, and relevant for this study, concern sepal identity, placentation topology and inflorescence architecture. Indeed in Arabidopsis, epidermal cell types and trichome architecture found on sepals can clearly be distinguished from those of leaves (Ditta et al., 2004). By contrast, petunia sepals display a similar kind of epidermal cell types as found in bracts and leaves, and are covered with the same type of multicellular trichomes (Figure 1C). While Arabidopsis sepals dehisce rapidly after fertilization of the flower and subsequently fall off together with petals and stamens, petunia sepals physiologically behave more as leaf-like organs: they stay firmly attached to the pedicel and may remain green, even long after the fruit has fully matured (Figure 1B). Note that the same occurs in flowers that were not fertilized (see further Figure 4F). The parietal placenta and ovules in Arabidopsis develop from the inner ovary wall, after termination of the floral meristem. In petunia, the central placenta arises directly from the center of the floral meristem in between the two emerging carpel primordia (Figure 1D), suggesting that the floral meristem is terminated later compared to Arabidopsis (Colombo et al., 2008). Finally, Petunia species develop a cymose inflorescence (Figure 1E, inset) as opposed to the raceme in Arabidopsis (reviewed in (Castel et al., 2010)). During petunia cymose inflorescence development, the apical meristem terminates by forming a flower, while an inflorescence meristem (IM) emerges laterally, repeating the same pattern (Souer et al., 1998). This results in the typical zigzag-shaped petunia inflorescence with alternating flowers on each node subtended by bracts.

Petunia SEP/AGL6 Expression Analysis and Mutant Identification
Six *SEP* genes and one *AGL6* gene (Ferrario et al., 2003; Vandenbussche et al., 2003b; Rijpkema et al., 2009) were described in petunia compared to 4 *SEP* genes and 2 *AGL6*-like genes in Arabidopsis. A survey of the recently released *Petunia axillaris* and *Petunia inflata* genome sequences (Bombarely et al., 2016) indicated that these sequences represent the total number of *SEP/AGL6* genes in petunia (Supplemental Table 1). Several detailed and robust phylogenetic studies of the *SEP* family (Zahn et al., 2005; Yu et al., 2016) as well as the more limited phylogenetic analysis presented here (Figure 1F), identified *FBP2* as the sole *SEP3* ortholog in petunia, meaning that the petunia *SEP3* clade contains only one member as in Arabidopsis. Petunia *FBP5* and *PMADS12 (PM12)* were shown to be the closest relatives of *SEP1* and *SEP2*, with the *FBP5/PM12* and *SEP1/SEP2* paralogous pairs originating from independent gene duplications in the lineages leading to petunia and Arabidopsis respectively. Finally, petunia *FBP4* grouped in the *SEP4* subclade, while *FBP9* and *FBP23* genes were members of the *FBP9* subclade, a subclass of *SEP* genes that is absent from the Arabidopsis genome and potentially may have been lost in the lineage leading to Arabidopsis (Malcomber and Kellogg, 2005; Zahn et al., 2005). The larger number of *SEP* genes in petunia compared to Arabidopsis is therefore entirely due to the presence of the two petunia *FBP9* subclade genes.

As expected based on their close taxonomic relationship, the petunia proteins overall showed the closest relationship with *SEP/AGL6* members from tomato (The Tomato Genome Consortium, 2012; Soyk et al., 2017) compared to Arabidopsis and rice (Figure 1F). Like petunia, tomato contained one *AGL6* gene, one *SEP3* copy and two *FBP9* members, but slight differences in the number of genes belonging to the *SEP1/SEP2* and *SEP4* subclades could be observed between the two species. Notably, tomato contained only one *SEP1/SEP2* copy, while having two *SEP4*-like genes. Among the members of the tomato *SEP/AGL6* family, the *SEP4*-like *RIN* gene initially received most of the attention, because the classical *rin* mutation has been widely used in tomato breeding as it improves shelf-life of tomato fruits when present in a heterozygous state, while the homozygous *rin* mutation prevents initiation of ripening (Vrebalov et al., 2002). Interestingly, more recent studies in tomato have shed a first light on the function of the enigmatic *FBP9* subclade genes. First of all, it was found that *SLMBP21/J2 (JOINTLESS 2)* is required for the development of the pedicel abscission zone (Liu et al., 2014; Roldan et al., 2017; Soyk et al., 2017). Furthermore, a breakthrough functional study (Soyk et al., 2017) based on both natural and CRISPR induced mutant alleles showed that the two tomato *FBP9* clade genes *SLMBP21/J2* and *SIMADS1/EJ2 (ENHANCER OF JOINTLESS 2)* have overlapping functions in meristem maturation and the control of inflorescence branching together with *LIN (LONG INFLORESCENCE)*, the second tomato *SEP4*-like gene.
Remarkably, triple *j2 ej2 lin* knockout mutants exhibit a dramatic phenotype consisting of massively overproliferated sympodial inflorescence meristems (SIMs) without the formation of flowers, indicating that the transition towards FM identity is not made.

As a first step in the characterization of the complete *SEP/AGL6* clade in petunia, we performed RT-qPCR expression analysis (Figure 1G) in three floral bud developmental stages (Figure 1E) with the stage 1 floral bud sample also including very early flower primordia, bracts and the inflorescence meristem, and in various other tissues. This allows for a more quantitative analysis than a previous study by RNA gel blot and *in situ* hybridization (Ferrario et al., 2003).

We detected important differences in expression levels among the *SEP/AGL6* genes and clear differences in expression patterns, both correlated with their phylogenetic position, suggesting functional divergence: *FBP2*, *FBP5*, and *AGL6* were the most abundantly expressed genes, reaching expression levels roughly tenfold higher than the *SEP4* homolog *FBP4*, and the *FBP9* subclade members *FBP9* and *FBP23*. Furthermore, *FBP2*, *FBP5* and *AGL6* expression was restricted to floral tissues, with expression levels strongly increasing during floral bud development, while *FBP4*, *FBP9* and *FBP23* were more broadly expressed, and expression levels did not show a strong upregulation during later stages of floral bud development. Expression outside the floral domain was most marked in bracts for *FBP4*, and in the inflorescence stem tissue for both *FBP4* and *FBP9*. One exception to these general differences observed between *SEP1/SEP2/SEP3/AGL6* and *SEP4/FBP9* genes was *PM12*, which was expressed ~100 fold lower than its close paralog *FBP5*, and for which expression was detected also in bracts and stems. For all genes analyzed, expression levels varied considerably between the different floral organs: Expression may be much lower in one particular organ type compared to the three other floral organs (e.g. very low *FBP2* and *FBP5* expression in sepals; low levels of *PMADS12, AGL6* and *FBP23* expression in stamens), or may peak in one specific floral organ (*FBP4* in sepals; *FBP9* in petals). Our results are in agreement with the *in situ* data previously obtained for *FBP2* and *FBP5*, showing mainly expression in the three inner floral whorls during early flower development, while some minor differences with the *PMADS12 in situ* data suggest that the *PM12* expression pattern is not constant as floral buds further develop (Ferrario et al., 2003).

To perform a functional analysis, we used a reverse genetics strategy (Koes et al., 1995; Vandenbussche et al., 2003b; Vandenbussche et al., 2008) to identify *dTph1* transposon insertions in the coding sequences of the petunia *SEP* and *AGL6* genes. In total, we identified and confirmed 16 independent transposon insertions *in planta*, including some earlier reported alleles (Vandenbussche et al., 2003b; Rijpkema et al., 2009) in all of the 7 different members
of the **SEP/AGL6** clade (Figure 1H). Because the 284 bp **dTph1** sequence encodes stop codons in all six possible reading frames, and based on their insert position (either disrupting the first exon encoding the MADS DNA binding domain, or the K-region required for protein-protein interactions in the case of the **fbp2** insertions, all of the selected insertion alleles most likely represent null alleles. We obtained and analyzed homozygous mutants for all insertion alleles, but remarkably, only homozygous mutants for **fbp2** insertions displayed floral homeotic defects (Figure 1H), suggesting extensive functional redundancy among the petunia **SEP/AGL6** genes, and that **FBP2** function is more essential than that of any other **SEP/AGL6** gene. These results clearly indicated the need for multiple mutant analyses to further uncover putative redundant functions.

**The Petunia **fbp2** **fbp5** **pmads12** Mutant, Genetic Equivalent of the Arabidopsis **sep1** **sep2** **sep3** Mutant, Displays Floral Characteristics Indicating Residual B- and C-Function Activity**

In Arabidopsis, the simultaneous loss of **SEP1**, **SEP2** and **SEP3** results in flowers consisting only of sepals (Pelaz et al., 2000). To compare the petunia genetic equivalent, we aimed to analyze **fbp2** **fbp5** **pm12** triple mutants (Figure 2). As mentioned earlier, of the three single mutants, only **fbp2** mutants displayed a phenotype different from WT (Figures 2A to 2D). Moreover, **fbp2/+** **fbp5** **pm12** flowers (Figure 2E) developed morphologically as WT, demonstrating that **FBP2** even in a heterozygous state can fully compensate for the loss of **FBP5** and **PM12** functions. In addition, **fbp2** **pm12** double mutants were not markedly different from **fbp2** single mutants (Figure 2F), in contrast to the earlier reported **fbp2** **fbp5** mutants (Figures 2G to 2K) and (Vandenbussche et al., 2003b). However, **fbp2** **fbp5** **pm12** flowers could be easily distinguished from **fbp2** **fbp5** flowers: a clear enhancement of stamen to sepal identity could be observed in the third whorl, although still some antheroid tissue remained, as in **fbp2** **fbp5** mutants (Figure 2M). Furthermore, while the extremely enlarged **fbp2** **fbp5** mutant pistil still exhibited partial carpel identity, the carpels of **fbp2** **fbp5** **pm12** mutants acquired clear sepal/leaf-like epidermal characteristics (Figures 2N to 2Q), and were densely covered with trichomes. The latter are never observed on WT pistils, and only at very low frequency on **fbp2** **fbp5** pistils (Figure 2N). Furthermore, no stigma and style structures remained in the triple mutant, but the overall internal organization of the ovary was maintained, with a placenta structure covered by a few hundred leaf-like organs that represented homeotically converted ovules, as observed in **fbp2** **fbp5** mutants (Figures 2K and 2L). In the second whorl of **fbp2** **fbp5** **pm12** flowers, the partial petal to sepal conversion at the corolla border was only subtly
enhanced compared to \textit{fbp2 fbp5} mutants (Figures 2G and 2H). Given that the effect of the \textit{pm12} mutation only became apparent in an \textit{fbp2 fbp5} mutant background, we conclude that \textit{PM12} plays a less essential role than its close paralog \textit{FBP5}. Overall, the remnant petal and stamen tissues and the maintenance of a placenta structure in \textit{fbp2 fbp5 pm12} flowers show that unlike in Arabidopsis, genes outside the \textit{SEP3} and \textit{SEP1/SEP2} subfamilies are able to rescue part of the B- and C-functions in a petunia \textit{sep1 sep2 sep3} mutant background.

The \textit{FBP9} Subclade Genes \textit{FBP9} and \textit{FBP23} Function as Floral Meristem Identity Genes together with \textit{FBP4}

We showed earlier that petunia \textit{AGL6} is one of the genes outside the classical \textit{SEP1/SEP2/SEP3} group that plays a prominent role in performing a SEP-like floral organ identity function, especially in the determination of petal identity, redundantly with \textit{FBP2} (Rijpkema et al., 2009). However, \textit{FBP4} as a \textit{SEP4}-like gene may also participate, as found in Arabidopsis (Ditta et al., 2004) and potentially also \textit{FBP9} and \textit{FBP23}, the petunia representatives of the \textit{FBP9} subclade.

Earlier we found that the \textit{fbp9 fbp23} and \textit{fbp4} single mutants displayed a WT phenotype (Figure 1H), and that expression levels of all three genes peak early during floral developmental stages compared to the other petunia \textit{SEP} genes and \textit{AGL6} (Figure 1G), potentially indicating a redundant (common) function for \textit{FBP4}, \textit{FBP9} and \textit{FBP23}. Indeed, a functional overlap was recently demonstrated among corresponding SEP subclade members in tomato (Soyk et al., 2017).

To test such a putative functional redundancy among the petunia \textit{FBP9}, \textit{FBP23} and \textit{FBP4} genes, we first created and analyzed \textit{fbp9 fbp23} double mutants, since \textit{FBP9} and \textit{FBP23} are close paralogs belonging to the same \textit{FBP9 SEP}-subclade. Interestingly, we found that \textit{fbp9 fbp23} mutants were dramatically affected in their inflorescence architecture, with new inflorescence shoots developing instead of flowers, resulting in a highly branched inflorescence structure. However, flower development was not completely abolished in these mutants, because after several weeks of a highly branched inflorescence development, frequently a flower appeared on one or more branches of the same plant, after which these branches switched again to the initial phenotype (Figures 3B and 3F). This indicated that the capacity to form floral meristems was not completely abolished in \textit{fbp9 fbp23} mutants and that (an)other factor(s) can partly rescue floral meristem determinacy in the absence of \textit{FBP9/FBP23} function. Because we assumed \textit{FBP4} being a likely candidate, we next analyzed \textit{fbp4 fbp9 fbp23} triple mutants. Indeed, we found that the \textit{fbp9 fbp23} phenotype was further enhanced in these triple mutants, resulting in a highly branched flowerless inflorescence architecture (Figures 3C, 3G
and 3H), phenotypically very similar to that reported earlier for the petunia floral meristem identity mutant alf (Souer et al., 1998), with ALF being orthologous to Arabidopsis LEAFY (LFY) (Weigel et al., 1992) and snapdragon FLORICAULA (FLO) (Coen et al., 1990) genes. Note that over a long period (> 6 months) of highly branched inflorescence development, some triple mutants produced 1-2 isolated flowers, while other individuals never flowered at all.

To study the fbp4 fbp9 fbp23 phenotype in more detail, we analyzed fbp4 fbp9 fbp23 inflorescence apices by scanning electron microscopy in comparison with WT (Figures 3I to 3L). At very early developmental stages, the fbp4 fbp9 fbp23 plants exhibited a phenotype very comparable to alf mutants (Souer et al., 1998): as in alf mutants, the bifurcation pattern of fbp4 fbp9 fbp23 inflorescence apices was similar to WT, but the two resulting meristems both behaved as inflorescence meristems, as indicated by the continuous bifurcation of each newly formed meristem and the repetitive formation of bracts flanking these meristems. Together, this indicates that floral meristems in fbp4 fbp9 fbp23 mutants are homeotically transformed into inflorescence meristems.

Finally, floral meristem identity was not visibly affected in fbp4 fbp9 fbp23/+ and fbp4 fbp23 fbp9/+ plants, as judged by the presence of a normal cymose inflorescence architecture in these mutant combinations (Figures 3M to O). This shows that the presence of either FBP9 or FBP23 in heterozygote state is sufficient to rescue floral meristem identity. Together with the already strong phenotype observed in fbp9 fbp23 plants compared to fbp4 fbp9 fbp23 plants (Figures 3B to 3C, 3F to 3G, 3P to 3Q), we conclude that the FBP9 clade members FBP23 and FBP9 play a major role in floral meristem identity determination in a largely redundant fashion, while FBP4 is involved in the same function, but plays a less essential role compared to the FBP9/FBP23 gene pair.

Although the phenotypes of tomato j2 ej2 lin and petunia fbp9 fbp23 fbp4 mutants at first sight do not look very similar (see discussion), overall, this shows that in both species, FBP9 clade genes together with a SEP4 gene play an essential role in floral meristem identity, different from the classical SEP organ identity functions.

To test whether FBP4, FBP9 and FBP23 also function later in conferring floral organ identity, we introduced the corresponding mutations into the fbp2 mutant background, the only petunia sep mutation with a visible phenotype as a single mutant. However, we found that flowers of fbp2 fbp4, fbp2 fbp9 and fbp2 fbp23 mutants were not markedly different from fbp2 mutants (Figures 3R to 3U), while fbp2 fbp4 fbp23 and fbp2 fbp4 fbp9 flowers only showed a moderate enhancement of the fbp2 petal-to-sepal conversion phenotype (Figure Figures 3V to 3W). In fbp2 fbp4 fbp23 flowers, the green margin appeared to be broader in all five petals.
while in \textit{fbp2 fbp4 fbp9} flowers this was most visible in the two basal petals. In comparison
with the earlier described floral phenotypes of \textit{fbp2 fbp5} (Vandenbussche et al., 2003b), \textit{fbp2 agl6, fbp2 fbp5 agl6} (Rijpkema et al., 2009) and \textit{fbp2 fbp5 pmads12} mutants, this suggests that \textit{FBP4, FBP9} and \textit{FBP23} do play a role in floral organ identity, but contribute only moderately

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The Sextuple \textit{fbp2 fbp4 fbp5 fbp9 pm12 agl6} Mutant Displays a Classic \textit{sepallata} Phenotype

By analyzing \textit{AGL6, FBP2} and \textit{FBP5} functions and the \textit{fbp2 fbp5 pmads12} and \textit{fbp4 fbp9 fbp23} triple and \textit{fbp2 fbp4 fbp9 fbp23} mutants, we could reveal specific/specialized SEP

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functions for certain members of the petunia \textit{SEP/AGL6} clade and surprisingly, the requirement

of \textit{FBP9/FBP23} function (and to a lesser extend \textit{FBP4}) for floral meristem identity, as was also

recently shown in tomato. However, a classic floral \textit{sepallata} phenotype as described for

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Arabidopsis was not obtained, indicating further redundancy, possibly shared between the

majority of the petunia \textit{SEP/AGL6} genes. To test this further, we embarked on a long-term
crossing scheme aimed to combine all of the \textit{sep/agl6} mutant alleles in a single plant. However,
we chose to exclude the \textit{fbp23} mutation in this scheme since this would completely abolish
flower formation when combined with the \textit{fbp9} and \textit{fbp4} mutations and thus prevent
visualization of additive floral phenotypes. After years of crossing, we finally obtained
homozygous sextuple \textit{fbp2-332 fbp4-44 fbp5-129 fbp9-90 pm12-37 agl6-118} mutant plants,
hereafter referred to as sextuple \textit{sep/agl6} mutants. In contrast to the earlier described lower
order mutants, all organs in the flowers of sextuple \textit{sep/agl6} mutants were green and densely
covered by trichomes (Figure 4), exhibiting sepal/leaf-like characteristics (Figures 4A to 4E).

Note that as mentioned earlier, it is not possible to discriminate between sepal, bract and leaf
identity in petunia based on epidermal cell characteristics (Figure 1C). As expected, scanning
electron microscopy of these organs revealed the conversion of the typical petal, stamen and
carpel epidermal cell types into epidermal cells characteristic for sepals, bracts and leaves,
including stomata and multicellular trichomes (Figure 4E). The second whorl, which in WT
consists of five large brightly colored fused petals, was occupied by five green organs that
remained fused at their bases (Figure 1C). Although dramatically smaller than WT petals, they
remained larger than first whorl sepals. Similarly, in the third whorl, the five stamens were
replaced by sepal/leaf like organs. The overall shape of these organs did retain some of the
stamen architecture, since the region corresponding to the WT stamen filament remained
smaller compared to the more leaf blade-like upperparts. Stamen filaments in WT are fused along half of their length with the inside of the petal tube. By contrast, third whorl organs in the sextuple mutant completely lost this partial fusion. In the fourth whorl, normally occupied by two carpels that are entirely fused and enclose the placenta and ovules, two (sometimes three) unfused sepal/leaf-like organs were found. Internally, the placenta was entirely replaced by a new emerging flower reiterating the same floral phenotype (Figure 1D). Thus in contrast to lower order sep mutants, the sextuple mutant was fully indeterminate. In the majority of the flowers (Figure 4F), this secondary flower further developed and emerged from the primary flower supported by a pedicel, while containing on itself another flower in its center. This third flower usually did not further grow out, although occasionally we observed up to three consecutive fully developed flowers (Figure 4F). Note that the sextuple sep/agl6 mutant displayed a normal cymose inflorescence architecture as in WT (Figure 4F), in sharp contrast to fbp4 fbp9 fbp23 mutants. This demonstrates that FBP23 alone can fully rescue floral meristem identity in a sextuple mutant background, but not floral organ identity.

**Homeotic Gene Expression in Sextuple sep/agl6 Mutant Flowers**

To further characterize the sextuple sep/agl6 mutant at the molecular level, we quantified and compared the dynamics of homeotic gene expression levels between WT and the sextuple mutant (Figure 4G) at three different stages of floral bud development, as described earlier (Figure 1E). Encoding of the B-function in petunia is more complex compared to Arabidopsis and Antirrhinum, and involves the two PI/GLO-like MADS-box transcription factors Petunia hybrida GLO1 and GLO2, the DEF/AP3 ortholog PhDEF, and PhTM6, the petunia representative of the ancestral B-class TM6 lineage that has been lost in Arabidopsis, but which is present in many species (Angenent et al., 1993; van der Krol et al., 1993; Vandenbussche et al., 2004; Rijpkema et al., 2006). In WT, we found that all four B-class genes were progressively upregulated as floral buds developed, with an upregulation from stage 1 to stage 3 varying roughly from three to six times, depending on the gene. In the sextuple mutant, we observed expression levels of PhGLO1, PhGLO2 and PhDEF initially similar to WT in the youngest stage analyzed. However, upregulation in older stages was strongly affected, especially for PhGLO1 and PhGLO2, which remained expressed at initial levels. PhDEF remained progressively upregulated in the different sextuple mutant samples, but reached only one third of the expression compared to WT in the final stage. By contrast, PhTM6 expression levels were strongly downregulated from stage 1 floral buds onwards, remaining at similarly low levels in the two older stages.
The C-function in petunia is redundantly encoded by $PMADS3$ and $FBP6$, orthologs of Arabidopsis AG and SHP1/2 respectively (Heijmans et al., 2012; Morel et al., 2018). As for the B-function genes, $FBP6$ and $PMADS3$ expression in WT is progressively upregulated in developing floral buds (6.5 and 11 times respectively), and initial expression levels in stage 1 buds were very similar between WT and sextuple mutants for both C-class genes. Both C-genes still displayed a clear upregulation in the older sextuple mutant flower buds, and seemed in general less affected by the sextuple loss of SEP/AGL6 function than the B-function genes, especially in stage 2. In stage 3 buds, $FBP6$ expression was not different between WT and sextuple mutants, while $PMADS3$ in the sextuple mutant was expressed at around 50% of its WT levels. $FBP11$ is a petunia D-lineage MADS-box gene orthologous to $STK$ (Angenent et al., 1995; Colombo et al., 1995), and that with $FBP7$ (another D-lineage member) and the C-genes $PMADS3$ and $FBP6$ redundantly is required to confer ovule identity, and to arrest the floral meristem (Heijmans et al., 2012). Consistent with its later function in floral development, the $FBP11$ expression profile showed a very strong upregulation in the WT developmental series (~30 fold). By contrast, in the sextuple mutant samples, $FBP11$ expression was barely detectable in all stages tested.

Finally, we choose to monitor the expression of petunia $UNS$ ($UNSHAVEN$), a member of the $SOC1$ subfamily, because of its particular expression pattern reported to be mainly restricted to green tissues including stems, leaves, bracts and the first whorl (sepals) in the flower (Immink et al., 2003; Ferrario et al., 2004). Moreover, $UNS$ ectopic expression was shown to confer leaf-like characteristics to floral organs. We first used the cDNA series from Figure 1F to analyze its expression pattern in a more quantitative manner compared to earlier gel blot data, confirming highest expression levels in bracts, inflorescence stems, and in the sepals within the flower (Supplemental Figure 1). In the WT developmental series, we found $UNS$ to be progressively downregulated as flower buds further developed, corresponding to a ~4 fold drop in expression levels compared to the youngest stage (Fig 4G). Interestingly, $UNS$ was expressed at higher levels in all sextuple mutant floral bud stages compared to WT, with the largest difference found in the oldest bud stage (~ 5-fold upregulation compared to WT). Moreover, a linear downregulation as in WT was not observed.

The Petunia $API/SQUA$ Subfamily: Phylogeny, Expression Analysis and Mutant Identification

We found that the petunia $SEP$ genes $FBP9$, $FBP23$ and $FBP4$ function primarily as floral meristem identity genes, a function which is in Arabidopsis mainly associated with
members of the AP1/SQUA MADS-box subfamily (Irish and Sussex, 1990; Mandel et al., 1992; Kempin et al., 1995; Ferrandiz et al., 2000). This raised the obvious question to what extent the petunia AP1/SQUA members are implicated in floral meristem identity determination. For these reasons, we aimed to functionally analyze the members of the petunia AP1/SQUA subfamily. Thus far, three petunia AP1/SQUA genes have been described, called PFG, FBP26 and FBP29 (Immink et al., 1999; Immink et al., 2003). In addition, based on sequence similarity, we identified a fourth AP1/SQUA member by the presence of an insertion mutant and associated transposon flanking sequence encountered in our transposon flanking sequence database, which we have called Ph-euAP1 (Petunia x hybrida euAP1), based on the presence of the highly conserved euAP1 motif (Litt and Irish, 2003; Vandenbussche et al., 2003a) in its C-terminus, as also found in the Arabidopsis AP1 and CAL genes (Supplemental Figure 2A). To provide further proof for the euAP1 classification of the new Petunia AP1/SQUA member, we conducted a phylogenetic analysis (Figure 5) including all AP1/SQUA subfamily members from Arabidopsis, tomato (Hileman et al., 2006; The Tomato Genome Consortium, 2012 and rice (Lee et al., 2003; Yu et al., 2016). Similar to the SEP/AGL6 phylogenetic analysis, overall the petunia proteins showed the closest relationship with AP1/SQUA members from tomato (Figure 5A), while all four rice AP1 members grouped apart from the eudicot proteins as shown previously (Yu et al., 2016). The analysis further showed that petunia euAP1 indeed is orthologous to the tomato MACROCALYX (MC) gene (Vrebalov et al., 2002) and the Arabidopsis AP1 and CAL genes, all previously demonstrated as belonging to the euAP1 clade (Litt and Irish, 2003; Yu et al., 2016). Petunia therefore is similar to tomato in having only one euAP1 clade member compared to two members in Arabidopsis. MC, the tomato euAP1 representative, was shown to regulate sepal size, fruit abscission and maintenance of inflorescence meristem identity. Indeed, mc mutants develop flowers with enlarged sepals, have an incomplete pedicel abscission zone, and develop inflorescences that revert to vegetative growth after forming two to three flowers (Vrebalov et al., 2002; Nakano et al., 2012; Yuste-Lisbona et al., 2016).

The previously described FBP29 gene fell into the AGL79 subclade to which the tomato genes MBP10 and MBP20 also belonged, while the PFG and FBP26 genes grouped into the euFUL subclade together with the tomato FUL1 (TDR4/TM4) and FUL2 (MBP7) genes as previously shown (Yu et al., 2016). While stable mutants remain to be described for these four tomato genes, RNAi mediated downregulation of FUL1 and FUL2 indicate a role for these genes in fleshy fruit ripening (Bemer et al., 2012; Shima et al., 2013; Wang et al., 2014). Furthermore, a role for MBP20 and FUL1 was proposed in the regulation of compound leaf
development (Burko et al., 2013). Finally, to date no function has been proposed for tomato MBP10 but an evolutionary study of the FUL genes in the Solanaceous family suggest that the MBP10 lineage, which is absent in petunia, may be undergoing pseudogenization (Maheepala et al., 2019). A sequence analysis of the Petunia axillaris and Petunia inflata genome sequences (Bombarely et al., 2016) further indicated that euAP1, PFG, FBP26 and FBP29 represent the total number of AP1/SQUA family members in petunia (Supplemental Table 1), similar to the size of the AP1/SQUA subfamily in Arabidopsis and rice, and one less compared to tomato (due to the absence of a MBP10 lineage member in petunia).

A quantitative expression analysis in different tissues and three floral bud developmental stages in WT (Figure 5B) showed that the expression patterns of the four genes were quite similar, although some minor differences did exist. Interestingly, expression levels of all four genes gradually decreased during floral bud development, suggesting an early developmental function, similar as what we observed for e.g. FBP9 and FBP4. Furthermore, during later flower development, moderate expression levels were detected in sepals, petals (except for FBP29) and carpels, while expression in stamens was considerably lower compared to the other floral organs. The four genes were also well expressed in inflorescence stem tissues as well as in bracts (with the exception of Ph-euAP1). Finally, PFG showed the broadest expression pattern, since moderate expression levels were also observed in vegetative apices and leaves. In addition, the peak values of PFG expression levels were around tenfold higher compared to those of Ph-euAP1, FBP26 and FBP29. The PFG expression data were in line with the broad expression pattern previously observed by RNA gel blot analysis and in situ hybridization (Immink et al., 1999), which revealed PFG expression in vegetative, inflorescence and floral meristems, in newly formed leaves, the vascular tissues, during early flower organ development and in carpel walls and ovules during later phases of pistil development.

To determine the function of the four petunia AP1/SQUA genes, we screened for dTph1 transposon insertions in their coding sequences, similarly as for the SEP genes. In total, we identified and confirmed 6 independent transposon insertions in planta (Figure 5C), including two earlier reported alleles (Vandenbussche et al., 2003b), potentially yielding putative null mutants for all four genes based on the insertion position of the dTph1 transposon, either disrupting the first exon encoding the MADS DNA binding domain or the K-region required for protein-protein interactions in the case of the euap1 allele. We obtained and analyzed homozygous mutants for all insertion alleles, but all these homozygous mutants developed normally (Figure 5C). Moreover, when we analyzed some double mutants to overcome putative
genetic redundancy, flowers in these double mutants developed normally, and inflorescence architecture was not affected (Supplemental Figure 2B).

**Petunia AP1/SQUA Family Members are Required for Inflorescence Meristem Identity**

Because of the absence of clear phenotypes in the above-described mutants, we decided to create and analyze pfg fbp26 fbp29 euap1 quadruple mutants (Figure 6). Remarkably, the flowers that developed on these quadruple mutants were fertile, and organ identity of the carpels, stamens and petals was not visibly affected (Figures 6A to 6C). However, sepals were considerably enlarged and contained sectors that exhibited homeotic conversion towards petal identity, as indicated by the red pigmentation and the presence of petal conical cells in these regions (Figure 6D). Overall, the general mildness of the pfg fbp26 fbp29 euap1 flower phenotype was very surprising, compared to the already dramatic phenotypes found in Arabidopsis ap1 single and ap1 cal double mutants, and compared to the complete absence of flowers in ap1 cal ful triple mutants (Irish and Sussex, 1990; Mandel et al., 1992; Kempin et al., 1995; Ferrandiz et al., 2000).

Quadruple pfg fbp26 fbp29 euap1 mutants did display a severe phenotype in inflorescence development. In fact, the normal cyme inflorescence architecture was completely abolished, and instead a large number of leaves were produced from the main apical meristem before terminating into a solitary flower (Figures 6E and 6G). In addition, branches that developed from the base of the plant followed exactly the same developmental pattern (Figure 6L). The leaves produced on the main stem and side branches were generated in a spiral phyllotaxy (Figures 6F and 6Q), characteristic of vegetative development (Figure 6P), in contrast to the opposite positioning of bracts in a WT inflorescence meristem. Finally, after the production of usually >25 leaves, this vegetative meristem was fully converted into a floral meristem resulting in a solitary flower (Figures 6H to 6I) as opposed to the normal cyme inflorescence structure in WT (Figure 6M). Note that quadruple mutant flowers consistently displayed an increase in floral organ number (e.g. the flower shown in Figure 6A has 10 petals), possibly because the full conversion of the vegetative meristem into a floral meristem resulted in a larger floral meristem size. In addition, the corolla of these flowers was not always properly organized, as the petal tube was often disrupted on one side.

Once this terminal flower was fully developed, new branches started to grow from vegetative meristems that were present in the axils of the leaves further down on the stem (Figure 6I). These branches produced again a large number of leaves before terminating with a solitary flower (Figure 6X), after which the same process was repeated. Together these results
indicate that petunia $AP1/SQUA$ genes are required to establish inflorescence meristem identity and associated cymose branching of the petunia inflorescence.

Interestingly, intermediate phenotypes could be observed in different triple mutants in which the fourth $AP1$ subfamily member was still in a heterozygous state (Figures 6S to 6Q), resulting in inflorescences in which each time several leaves developed before the next flower-bearing node was produced. Together this indicates that all four genes contribute to cymose inflorescence development in petunia.

**Petunia $AP1/SQUA$ Family Members Repress the B-Function in the First Whorl in Concert with the ROB/BEN Genes.**

The partial sepal-to-petal homeotic conversion in flowers of $pfg fbp26 fbp29 euap1$ mutants (Figure 6D) suggests that petunia $AP1/SQUA$ genes negatively regulate the B-function in the first floral whorl. Recently we demonstrated that the $AP2$-type $REPRESSOR$ OF $B$ ($ROB1$), $ROB2$ and $ROB3$ genes repress the B-function in the first whorl, together with $BEN$, a TOE-type $AP2$ gene (Morel et al., 2017). To further explore the implication of the petunia $AP1/SQUA$ genes in patterning the B-function, we tested their genetic interaction with $ROB$ genes. We crossed $pfg fbp26 fbp29 euap1$ and $rob1 rob2 rob3$ mutants and screened progenies for an enhanced sepal-to-petal homeotic conversion phenotype compared to $pfg fbp26 fbp29 euap1$ and $rob1 rob2 rob3$ mutants. Among a large progeny, we found individuals displaying the $pfg fbp26 fbp29 euap1$ inflorescence phenotype while bearing terminal flowers of which the first whorl organs showed a much more pronounced sepal-to-petal conversion compared to $pfg fbp26 fbp29 euap1$ mutants. We genotyped several of these plants for the seven insertions, and found that plants with the strongest phenotype were $rob1 rob2/+ rob3 pfg fbp26 fbp29 euap1$ (Figures 6J and 6K). Flowers of these mutants had first whorl organs that clearly formed the beginning of a petal tube (Figure 6J), although not fused along its entire length, and with strongly expanded petaloid regions compared to the first whorl organs of $pfg fbp26 fbp29 euap1$ flowers (Figures 6D and 6K). The presence of pale pigmentation at the basal end of the organs and bright red at the distal end (Figure 6K) was also characteristic for the modular tube/corolla architecture of a WT petunia petal (Figures 1A and 2I). For comparison, first whorl sepals of $rob1 rob2/+ rob3$ plants had a phenotype similar to WT (Figures 6N to 6O), while $rob1 rob2 rob3$ flowers exhibit a very subtle sepal-to-petal conversion at the margins of their sepals, and which is only clearly visible in the first 2–3 flowers that develop (Morel et al., 2017). Although we did not obtain plants homozygous for all seven mutations, the synergistic interaction observed between $rob1 rob2/+ rob3$ and $pfg fbp26 fbp29 euap1$ mutations strongly supports a
role for petunia $API/SQUA$ genes in repressing the B-function in the first whorl, together with
the $ROB/BEN$ genes.

**DISCUSSION**

A Comparison of $SEP/AGL6$ and $API/SQUA$ Functions in Petunia, Arabidopsis, Tomato
and Rice

In this study, we exploited the natural $dTph1$ transposon mutagenesis system in
petunia to identify mutants for all 11 members of the petunia $API/SEP/AGL6$ superclade, and
created a series of higher order mutants to uncover putative redundant functions. Here we
discuss and compare our findings with the available functional data from mainly Arabidopsis,
tomato and rice (see Figures 1F and 5A for the composition of their $SEP/AGL6$ and $API/SQUA$
subfamilies). Petunia and tomato on the one hand, and Arabidopsis on the other hand are
representatives of the Asterids and Rosids respectively, which constitute the two major groups
in the core eudicots, and are thought to have diverged $>100$ million years ago (Moore et al.,
2010). Comparison of the molecular mechanisms controlling flower development in these
species therefore helps to assess conservation and divergence of the floral regulatory gene
network in the core eudicots (Vandenbussche et al., 2016). Petunia and tomato both belong to
the Solanaceous family, and the lineages leading to petunia and tomato are estimated to have
diverged around 30 MYA (Bombarely et al., 2016). Their close relationship is indeed reflected
in a high degree of sequence similarity between tomato/petunia orthologous pairs in the
$AP1/SEP/AGL6$ superclade (see also Supplemental Data Files 1, 2, 3 and 4), which makes the
petunia/tomato comparison particularly well suited to evaluate functional diversification
patterns on a shorter evolutionary time-scale, as opposed to the comparison with the distant
monocot model species rice.

Implication of $SEP$ and $AGL6$ Gene Functions in Floral Organ Identity

Our genetic analysis in petunia indicates that its $SEP3$ ortholog $FBP2$ encodes the major
$SEP$ organ identity function: $FBP2$ is capable of fully rescuing flower development in a $fbp2/+$
$fbp5$ $pm12$ mutant background, and $fbp2$ is the only $sep$ single mutant with a clearly visible
phenotype. In Arabidopsis, all available genetic data indicate that $SEP3$ is also the most
important $SEP$ gene. Indeed, it was reported that single $sep3$ mutants display a phenotype on
their own, showing a mild petal to sepal conversion, while $sep1$, $sep2$ and $sep4$ single mutants
showed no developmental abnormalities (Pelaz et al., 2001). Secondly, $sep1$ $sep2$ $sep4$ mutants
show no significant perturbation of floral organ development, indicating that $SEP3$ can fully
rescue WT development in a triple mutant background (Ditta et al., 2004). Thus SEP3 seems to perform a master SEP floral organ identity function in both species.

While the gene-silencing approaches used to analyze SEP3 function in tomato and rice do not yet allow such detailed conclusions, these experiments suggest that their SEP3 orthologs play also a major role in floral organ identity: Tomato TM5 co-suppression lines genes exhibited homeotic conversion of whorls 2, 3, and 4 into sepal-like organs and loss of determinacy in the center of the flower (Pnueli et al., 1994) and a Y2H study found that TM5 was the preferred bridge protein of the 5 SEP tomato proteins tested (Leseberg et al., 2008). Transgenic lines carrying a construct aimed at simultaneously downregulating the two SEP3-like rice OsMADS7 and OsMADS8 genes were late flowering, and carried flowers exhibiting partial homeotic conversions of the floral organs in the three inner whorls into palea/lemma-like organs, and a partial loss of floral determinacy (Cui et al., 2010).

Arabidopsis sep1 sep2 sep3 mutants display a full conversion of petals, stamens and carpels into sepals, and flowers are fully indeterminate (Pelaz et al., 2000). By contrast, the genetically equivalent fbp2 fbp5 pmads12 mutant in petunia retains -albeit reduced- petal and stamen tissues, and the basic organization of the placenta structure in the flower center is maintained. Thus unlike in Arabidopsis, genes outside the SEP3 and SEP1/SEP2 clades are able to rescue part of the B- and C-functions in a petunia sep1/sep2/sep3 mutant background. We identified petunia AGL6 as one of these genes (Rijpkema et al., 2009). Similarly, the two rice AGL6 genes OsMADS6/MFO1 and OsMADS17 perform SEP-like functions, partly in a redundant fashion with the SEP gene OsMADS1/LHS1 (Ohmori et al., 2009; Dreni and Zhang, 2016). More recently, a floral organ identity function was also proposed for the tomato AGL6 gene, based on RNAi (Yu et al., 2017). Despite that the Arabidopsis genome encodes two AGL6 homologs (AGL6 and AGL13), the phenotype of the sep1 sep2 sep3 mutant demonstrates that Arabidopsis AGL6 genes may have lost most of their SEP-like activity compared to petunia, rice and tomato AGL6 genes, in agreement with the diverse proposed functions for Arabidopsis AGL6 and AGL13 (Koo et al., 2010; Huang et al., 2012; Hsu et al., 2014).

Furthermore, we showed that in a petunia sextuple sep/agl6 mutant a full sepallata phenotype was obtained, including complete loss of floral meristem termination. Remarkably, the obtained phenotype was similar to that of the earlier described FBP2 co-suppression line (Ferrario et al., 2003), demonstrating the efficiency of co-suppression to silence multiple genes simultaneously. The expression levels of all six petunia SEP genes (but not of AGL6) were monitored in the co-suppression line, but only FBP2 and FBP5 were found to be
downregulated. This strongly suggests that other genes were silenced at the post-transcriptional
level as was reported to frequently occur in gene silencing experiments (Stam et al., 1997).
Measuring mRNA levels of paralogous genes thus appears to be a limited method to assess the
specificity of a silencing construct.

The addition of the sep4 mutation to the Arabidopsis triple sep1 sep2 sep3 mutant
resulted in the conversion of sepal-like organs into leaf-like organs, indicating that SEP genes
are required to specify sepal identity (Ditta et al., 2004). The fact that we could not observe a
transition from sepal towards leaf-identity in the sextuple sep/agl6 mutant is most likely directly
related to the 'leaf'-like identity of petunia WT sepals. Such basic differences in sepal identity
between Arabidopsis and other species such as petunia may be contributing to the difficulties
to formulate a broadly applicable A-function (Litt, 2007; Causier et al., 2009).

Transgenic lines in which at least four of the rice SEP-like genes (OsMADS1/LHS1
(LEAFY HULL STERILE1)), OsMADS5, OsMADS7 and OsMADS8) were downregulated,
showed homeotic transformation of all floral organs except for the lemma into leaf-like organs
(Cui et al., 2010), reminiscent of the Arabidopsis sep1 sep2 sep3 sep4 quadruple mutant flower
phenotype. Remarkably however, severe loss-of-function mutations in the LOFSEP gene
OsMADS1/LHS1 alone can cause complete homeotic conversion of organs of the three inner
whorls into lemma/palea-like structures, and loss of floral meristem determinacy (Agrawal et
al., 2005), while also dominant-negative and milder phenotypes were reported for other
OsMADS1/LHS1 alleles (Jeon et al., 2000; Chen et al., 2006). More recently, Wu and
colleagues specifically investigated unique and redundant functions of the three LOFSEP genes
using mutant alleles and found that OsMADS1/LHS, OsMADS5, and OsMADS34/PAP2
(PANICLE PHYTOMER2) together regulate determinacy of the floral meristem and specify the
identities of spikelet organs by positively regulating the other MADS-box floral homeotic genes
including B-, C-, SEP3 and AGL6 genes (Wu et al., 2017a).

In petunia sextuple sep/agl6 mutant flowers, we found that the initial expression levels
of the B-class genes PhGLO1, PhGLO2 and PhDEF and of the C-class genes PMADS3 and
FBP6 were comparable to WT, indicating that initial activation and expression of these genes
does not depend on the SEp/AGL6 floral organ identity function. In Arabidopsis, a similar
observation has been made, showing normal patterning and accumulation of AP3, PI and AG
expression in young sep1 sep2 sep3 floral buds (Pelaz et al., 2000). With perhaps the exception
of FBP6 (SHP1/2), we found that further upregulation during later stages of development was
impaired, especially for the PI homologs PhGLO1 and PhGLO2, while PhDEF (AP3) and
PMADS3 (AG) still showed upregulation, but with a smaller incremental rate. These results are
in agreement with the idea that in Arabidopsis, complex formation of SEP proteins with B- and C-class MADS-box proteins is required for their positive autoregulation (Gomez-Mena et al., 2005; Kaufmann et al., 2009).

In sharp contrast with the other B-class genes, *PhTM6* expression levels in the sextuple mutant were almost completely abolished during all stages tested, indicating a full dependence on SEP/AGL6 activity for all stages of its expression. Earlier, we showed that regulation of *PhTM6* expression is atypical for a B-class gene, since its expression largely depends on the activity of the C-genes *PMADS3* and *FBP6* (Heijmans et al., 2012), resulting in a WT expression pattern mainly in stamens and carpels from early developmental stages onwards, and in all floral whorls when the C-genes are ectopically expressed (Vandenbussche et al., 2004; Rijpkema et al., 2006). Together, this indicates that both SEP and C-class genes are absolutely required for *PhTM6* expression, most likely as interaction partners in a MADS-box protein complex (Ferrario et al., 2003). For *FBP11* (STK) expression, we found the same SEP dependence, but since *FBP11* is expressed relatively late during flower development in the developing placenta and ovules (Angenent et al., 1995), this may also be an indirect effect, since these tissues are completely absent in the sextuple mutant. Thus, B- and C-class MADS-box proteins may have an absolute requirement of SEP function to activate their downstream developmental programs, but depend only partly on it for upregulation of their own expression. This suggests differences in the molecular mechanisms involved in autoregulation versus downstream target gene activation/repression.

Finally, we found that *UNS*, a petunia member of the *SOC1* family, was strongly upregulated in the sextuple *sep/agl6* mutant from early stages onwards, suggesting that SEP genes repress *UNS* during WT flower development. *SOC1* was identified as a direct target of SEP3 in a genome wide study in Arabidopsis, with the expression of *SOC1* being already reduced after only 8h of *SEP3* induction in seedlings (Kaufmann et al., 2009). Interestingly, it was shown that constitutive *UNS* expression in petunia and Arabidopsis flowers lead to the unshaven floral phenotype, which is characterized by ectopic trichome formation on floral organs and conversion of petals into organs with leaf-like features (Ferrario et al., 2004). All these observations are consistent with the finding of Ó’Maoileidigh and colleagues, who demonstrated that the floral homeotic organ identity gene *AG* not only functions by positively conferring floral identity to organ primordia in the flower, but also by actively repressing components of the leaf developmental program (Ô’Maoileidigh et al., 2013).
The FBP9 Subclade Genes together with a SEP4-like Gene are Required to Confer Floral Meristem Identity in petunia and tomato.

We found that the FBP9 subclade members FBP9 and FBP23 together with FBP4 play a crucial role in floral meristem identity, as illustrated by the homeotic transformation of flower meristems into inflorescence meristems in \textit{fbp9 fbp23 fbp4} triple mutants. In contrast, genetic interactions with the \textit{fbp2} mutant revealed only mild contributions to the classical SEP organ identity function. The phenotype of the \textit{fbp9 fbp23 fbp4} triple mutant is strikingly similar to that of the floral meristem identity mutants \textit{alf} and \textit{dot} (Souer et al., 1998; Souer et al., 2008), but it remains to be investigated how these genes are hierarchically positioned. However, it was found that simultaneous overexpression of \textit{ALF} and \textit{DOT} in young seedlings led to strong activation of \textit{FBP9} and \textit{FBP23} expression (Souer et al., 2008), suggesting that ALF/DOT specify floral meristem identity at least in part by activating \textit{FBP9} and \textit{FBP23} expression. An expression analysis of \textit{ALF, DOT, FBP9, FBP23} and \textit{FBP4} in the different mutant backgrounds may provide further support for this hypothesis.

Importantly, our analysis of the \textit{fbp4 fbp9 fbp23} mutant combined with a recent study of tomato FBP9 and SEP4 subclade members (Soyk et al., 2017) demonstrates that the requirement of \textit{FBP9} and \textit{SEP4} clade genes for floral meristem identity is conserved between tomato and petunia, and therefore likely also in other Solanaceous species. Note that although in both cases floral meristem identity is compromised, the phenotypes of tomato \textit{j2 ej2 lin} and petunia \textit{fbp9 fbp23 fbp4} mutants superficially do look quite different. We believe that this may be explained for an important part by basic differences in the inflorescence architecture between the two species. First of all, in petunia, every flower arises from a node that bears two bracts, while the tomato inflorescence is bractless. As a consequence, loss of FM identity in petunia leads to a highly branched structure composed of a lot of bracts, while in tomato this leads to a more naked structure consisting of proliferating SIMs. Also, the compound tomato inflorescence architecture is more complex compared to petunia and involves the transition of a vegetative meristem into a transition meristem (TM) that terminates in a floral meristem (FM) resulting in the first flower of the inflorescence. Additional flowers then develop from the axillary SIM, resulting in an inflorescence bearing multiple flowers (Park et al., 2014).

While the strongest phenotype was obtained in the tomato triple mutants, analysis of single and double mutants revealed also individual contributions to tomato development: \textit{LIN} limits internode length and the number of flowers that develop per inflorescence, \textit{EJ2} negatively regulates sepal size, while both \textit{J2} and \textit{EJ2} are involved in the control of branching
of the tomato inflorescence (Soyk et al., 2017). In addition, J2 is required for the development
of the pedicel abscission zone (Liu et al., 2014; Roldan et al., 2017; Soyk et al., 2017).

Finally, remark that our phylogenetic analysis indicates that within the SEP4 clade, RIN
in fact is more closely related to petunia FBP4 compared to LIN. However, RIN shows a much
more restricted expression pattern limited to the developing fruit (Vrebalov et al., 2002),
indicating that RIN has evolved a specialized role compared to FBP4 and LIN. Because of the
rin phenotype, RIN has long time been considered to function as a major regulator that is
essential for the induction of ripening, but a recent study using a CRISPR/Cas9-mediated RIN-
knockout mutation shows that inactivation of RIN does not repress initiation of ripening and
that the original rin mutation is rather a gain-of-function mutation resulting in an aberrant
protein that actively represses ripening (Ito et al., 2017).

While Arabidopsis doesn’t have FBP9 subclade members (Zahn et al., 2005), it was
found that Arabidopsis SEP proteins, in addition to their role in floral organ identity, are also
involved in maintaining floral meristem identity, as evidenced by the frequent appearance of
secondary flowers in the axils of first-whorl organs in sep1 sep2 sep3 sep4 quadruple mutants
and much less frequently in sep1 sep2 sep 3 mutants (Ditta et al., 2004). Moreover, an ap1 sep1
sep2 sep4 quadruple mutant was shown to produce a cauliflower phenotype similar to ap1 cal
mutants, while an ap1 sep4 mutant had a meristem identity defect intermediate between that of
ap1 and ap1 cal mutants. Although these data clearly demonstrate the implication of
Arabidopsis SEP genes in floral meristem identity, the very severe floral meristem defects
observed in ap1 cal or ap1 cal ful mutants, indicate that in Arabidopsis, floral meristem is
mainly determined by members of the AP1/SQUA subfamily.

In rice, the three LOFSEP genes OsMADS1/LHS, OsMADS5, and OsMADS34/PAP2
were proposed to be involved in the transition of the spikelet meristem into a floral meristem
(Wu et al., 2017a). However, floral meristem formation in the triple osmads1 osmads5
osmads34 mutants was not completely abolished, only strongly delayed, possibly because the
insertion alleles are not complete null mutants as suggested by the authors (Wu et al., 2017a).

The Petunia AP1 ortholog euAPI is not required for petal development, and acts
redundantly with the other API clade members as a B-function Repressor in the First
Floral Whorl.

Because Arabidopsis ap1 mutants lack petals and have sepals displaying bract like
features (Irish and Sussex, 1990; Mandel et al., 1992) and AP1 is negatively regulated by AG
in whorls three and four (Gustafson-Brown et al., 1994), *AP1* has been classified as an A-function gene in the ABC model, required for the identity specification of sepals and petals. In sharp contrast with the phenotype of Arabidopsis *ap1* mutants, we found that petal development does not at all require eu*AP1* activity in petunia. This may not come as a complete surprise since it was shown before that also in Arabidopsis, *AP1* is not essential for petal development, as evidenced by the nearly complete restoration of petal development in *ap1 ag* mutants (Bowman et al., 1993) and in *35S:SEP3 ap1* flowers (Castillejo et al., 2005), and a partial restoration in *ap1 agl24* double mutants (Yu et al., 2004). In addition, single eu*ap1* mutants that still develop petals have previously been described in other species such as e.g. the *squa* mutant in snapdragon (Huijser et al., 1992), the *pim* mutant in pea (Berbel et al., 2001; Taylor et al., 2002), *mtap1* in *Medicago* (Benlloch et al., 2006; Cheng et al., 2018), and *mc* in tomato (Vrebalov et al., 2002; Nakano et al., 2012; Yuste-Lisbona et al., 2016).

Restricting the activity of the floral homeotic B- and C-functions to their proper domains is crucial for the correct development of the flower structure, and it appears that the molecular mechanisms underlying these cadastral functions are much more diverse compared to the floral organ identity functions (reviewed in (Monniaux and Vandenbussche, 2018)). Here we identified the petunia *AP1/SQUA* genes as repressors of the B-function in the first whorl, as evidenced by the partial conversion of sepals into petaloid tissue in *pfg fb*26 *fb*29 eu*ap1* mutants, and the strong enhancement of this phenotype in combination with mutations in the *ROB* genes, which were previously identified as B-function repressors in the first whorl (Morel et al., 2017). Such a phenotype has so far never been reported in flowers of Arabidopsis *ap1*, *cal* or *ful* mutants, or any combination of these mutations (Ferrandiz et al., 2000). Nevertheless, it was proposed that *AP1* in combination with *AGL24* (*AGAMOUS LIKE 24*) and *SVP* (*SHORT VEGETATIVE PHASE*) represses both the B- and C-function genes during early phases of floral development (Gregis et al., 2006; Gregis et al., 2009), but it is not clear if other Arabidopsis *AP1/SQUA* genes would be also involved in this process and whether this is specific to the first floral whorl. Finally in rice, deregulation of B- and C-expression patterns was observed in *osmads14 osmads15/+* and *osmads14/+ osmads15* flowers (Wu et al., 2017b), suggesting that these rice AP1/SQUA transcription factors are also involved in patterning the homeotic B- and C-functions.

In summary, the observation that the petunia *AP1/SQUA* genes repress the B-function in the first floral whorl but do not seem to be required for 2nd whorl petal development demonstrates that petunia *AP1/SQUA* genes cannot be easily classified as “A-function” genes according to the original definition of the A-function in the ABC model. Earlier, we
encountered the same difficulties when trying to integrate the function of the petunia AP2-like transcription factors AP2 and ROBI-3 into a simple ABC model (Morel et al., 2017). This led us to propose a modified model for petunia floral organ identity in which the original A-function is replaced by a combinatorial function describing the cadastral (boundary setting) mechanisms that pattern the floral B- and C-functions (Morel et al., 2017). The above described cadastral function of the petunia API/SQUA genes during flower development perfectly fits into this alternative model, and is also compatible with the proposed modified (A)BC model (Causier et al., 2009), in which a more broadly defined (A)-function provides the genetic context in which the B- and C-functions are active and regulates both their spatial and temporal expression domains. Our findings for both the API/SQUA and AP2-like gene functions in petunia entirely explain the struggles to translate the Arabidopsis definition of the A-function to distant flowering species (Litt, 2007).

Petunia API/SQUA Family Members Function in a Largely Redundant Fashion and are Required for Inflorescence Meristem Identity.

Different members of the API/SQUA subfamily in Arabidopsis have evolved unique roles during development as exemplified by the distinct phenotypes of the single ap1 and ful mutants. Swapping experiments suggest that functional divergence between AP1 and FUL is due to changes in both expression pattern and coding sequence (McCarthy et al., 2015). At the same time, AP1, CAL and FUL have retained a redundant function in inflorescence architecture (Ferrandiz et al., 2000), whereas CAL shares a cryptic role in petal development redundantly with AP1 (Castillejo et al., 2005). While the function of AGL79 (a euFUL-like gene) has remained elusive for a long time, a recent study suggests a role for AGL79 in lateral root development and control of lateral shoot branching (Gao et al., 2017). It remains to be established if AGL79 overlaps in function with AP1, CAL and FUL.

Although we cannot exclude to have overlooked some very subtle defects, the absence of clear floral developmental defects in mutants for any of the four petunia API/SQUA genes suggests that individual members of the petunia API/SQUA subfamily did not functionally diverge, independent from their euAP1 or euFUL/paleoAPI clade identity. In line with that, we found that all four genes show overlapping expression patterns in most tissues tested. It remains to be tested whether this broad functional redundancy is also observed during other developmental processes, such root or fruit development, which were not analyzed in this study.

One of the striking aspects of the phenotype of quadruple pfg fbp26 fbp29 euap1 mutants is that these plants develop fully functional flowers, suggesting that floral meristem identity
does not require AP1/SQUA activity in petunia. Our finding that this function is apparently
taken care off by a specific subset of SEP genes fully fits this hypothesis. However, we can
currently not fully exclude that some residual AP1/SQUA activity remains in the pfg fbp26
fbp29 euap1 mutants, possibly explaining the formation of terminal flowers. Especially the pfg-
12 insertion allele potentially could be a hypomorphic allele, since an alternative startcodon is
present in the first exon (AA nr 8, Supplemental Data File 2) shortly after the transposon
insertion site. This could in theory lead to the production of a protein with an N-terminal
truncation of the MADS-domain, perhaps displaying some residual functionality. Other alleles
will have to be identified in the future to fully proof the hypothesis that floral meristem identity
in petunia does not require AP1/SQUA activity.

On the other hand, the phenotype of the quadruple pfg fbp26 fbp29 euap1 mutants
indicate that the petunia AP1/SQUA genes appear to be essential for the development of the
cymose inflorescence, indicating a role in inflorescence meristem identity. Such a role also has
been proposed for AP1/SQUA genes in other core eudicot species: VEG1 and its ortholog
MtFUL are essential for the specification of the secondary inflorescence meristem in the
compound inflorescences of pea and Medicago respectively, but are not required for floral
meristem identity (Berbel et al., 2012; Cheng et al., 2018).

Interestingly, it was earlier found that the tomato mc mutants also play a role in
inflorescence meristem development, since mc inflorescences revert to vegetative growth after
forming two to three flowers. In addition, these flowers developed enlarged sepals and have an
incomplete pedicel abscission zone (Vrebalov et al., 2002; Nakano et al., 2012; Yuste-Lisbona
et al., 2016). Moreover, the implication of MC in the development of the pedicel abscission
zone is proposed to occur via a higher order MADS-box complex including the SVP-like
protein JOINTLESS (J), and J2/SLMBP21 a SEP FBP9 clade member. Except for the pedicel
abscission zone which does not exist in petunia, the mc phenotypes are reminiscent of what we
observed in petunia quadruple ap1 mutants, suggesting a conserved role in inflorescence
meristem identity and first whorl development. Because mc single mutants have a clear
phenotype on their own, it also shows that MC exhibits less functional overlap with the other
AP1 family members compared to petunia. However, as suggested by the relative mildness of
the inflorescence meristem defect in mc mutants compared to the petunia quadruple mutants,
this does not exclude possible partial redundancy with one or more of the other tomato AP1
family members, something that still remains to be tested. RNAi mediated downregulation of
tomato FUL1 and FUL2 suggested a role for these genes in fleshy fruit ripening (Bemer et al.,
2012; Shima et al., 2013; Wang et al., 2014), indicating that the implication of FUL genes in
fruit development is conserved between tomato and Arabidopsis, despite that these two species have very different fruit types (fleshy versus dry). Petunia on the other hand develops a dry fruit capsule, but the implication of AP1/FUL members in its development remains to be investigated.

Finally, of the four identified rice AP1 subfamily members called OsMADS14, OsMADS15, OsMADS18 and OsMADS20 (Lee et al., 2003; Yu et al., 2016), it was found that OsMADS14, OsMADS15 and OsMADS18 are specifically activated in the meristem at phase transition together with the LOFSEP gene PAP2/OsMADS34 (Kobayashi et al., 2010; Kobayashi et al., 2012). While downregulation of these three AP1/FUL-like genes by RNAi caused only a slight delay in reproductive transition, further depletion of PAP2 function from these triple knockdown plants inhibited the transition of the meristem to the IM (Kobayashi et al., 2012), indicating that the AP1/FUL-like OsMADS14, OsMADS15, OsMADS18 and the LOFSEP gene PAP2/OsMADS34 coordinately act in the meristem to specify inflorescence meristem identity. In addition, it was shown that OsMADS14 and OsMADS15, besides to their function of specifying meristem identity, are also involved in the specification of palea and lodicule identities, using stable mutant alleles (Wu et al., 2017b).

**Functional Diversification Patterns in the AP1/SEP/AGL6 Superclade during Angiosperm evolution.**

Above, we compared AP1/SEP/AGL6 functions between different species, mainly focusing on Arabidopsis, petunia, tomato and rice, revealing important differences in the functions performed by their respective members. Perhaps the most striking observation is that a subclass of SEP genes (all belonging to the LOFSEP group) in petunia, tomato and possibly also rice are required to confer floral meristem identity, while in Arabidopsis the floral meristem identity function is mainly associated with members of the AP1/SQUA subfamily. It thus seems that during angiosperm evolution, members of different subfamilies within the AP1/SEP/AGL6 superclade have evolved specialized/subfunctionalized roles either in floral organ identity or inflorescence and/or floral meristem determination, providing further genetic support for the monophyletic origin of the AP1/SEP/AGL6 superclade. Within MADS-box subfamilies, it is not unusual that functions have been distributed differently between paralogs in different species. One of the first well documented cases concerns the C-function MADS-box subfamily, showing that the canonical C-function is encoded by nonorthologous genes in Arabidopsis and Antirrhinum (Causier et al., 2005). However, careful comparison of gene functions in the AP1/SEP/AGL6 superclade suggest that this random distribution of functions after gene
duplication has occurred also during the earlier phases of the evolution of the MADS-box gene family, resulting in functions that are differently distributed beyond the subfamily level. In addition, comparison between tomato and petunia indicates major functional differences that have arisen on a relatively short evolutionary time-scale. Of note is the involvement of several tomato \( \text{API/SEP/AGL6} \) members in the development of the pedicel abscission zone and in compound leaf development, all processes that do not occur in petunia.

Together, these observations illustrate that gene function cannot accurately be predicted solely based on sequence homology and phylogenetic analysis, and that final gene function may be strongly dependent on species-specific developmental contexts. Furthermore, it also illustrates that demonstration of gene function conservation between only two species, even if they are very distantly related (e.g. a monocot versus a dicot species), cannot safely be used to extrapolate a more general conservation of a particular gene function. Together with other studies, this further enforces the argument that plant biology in general, and plant evo–devo in particular would strongly benefit from a broader range of available model systems (Vandenbussche et al., 2016).

**MATERIALS AND METHODS**

**Plant Material, Genotyping and Phenotyping**

Petunia plants were grown in soil (FAVORIT-argile 10) either in a greenhouse (16 h day/8 h night: natural light supplemented with Philips Sodium HPS 400W SON-T AGRO light bulbs; 55,000 lumens) or outside protected by an agricultural tunnel (from April to October), both under conditions that depend on local seasonal changes (45.72°N 4.82°E), or in growth chambers (settings: 16 h day 22°C /8 h night 18°C, 75W Valoya NS12 LED bars, light intensity 130 \( \mu \)E). The identification of the following \( dTph1 \) transposon insertion alleles (Figures 1G and 5D) was described previously (current allele naming based on exact insert position; old allele names in between brackets): \( fbp2-332 \) (\( fbp2-1 \)); \( fbp2-440 \) (\( fbp2-2 \)); \( fbp4-44 \) (\( fbp4-2 \)); \( fbp4-55 \) (\( fbp4-3 \)); \( fbp5-129 \) (\( fbp5-1 \)); \( fbp9-110 \) (\( fbp9-1 \)); \( pfg-12 \) (\( pfg-1 \)); \( fbp26-76 \) (\( fbp26-1 \)) (Vandenbussche et al., 2003b), and \( agl6-118 \) (\( agl6-1 \)) (Rijpkema et al., 2009). Note that the previously determined insert positions for some of these alleles differ by a few nucleotides compared to the data presented here, due to the imperfect manual sequencing method used at that time combined with the characterization of only the right border of the transposon flanking sequences, not taking the \( dTph1 \) 8bp target site duplication into account. Also, it was mentioned that homozygous \( fbp9-1 \) (\( fbp9-110 \)) mutants exhibited aberrations in plant architecture during the reproductive phase (Vandenbussche et al., 2003b). However, later outcrossing analysis of
the fbp9-1 allele showed that this defect was closely linked to fbp9-1
insertion, as confirmed by the absence of this phenotype in the new fbp9-7 and fbp9-90 alleles.
The following alleles fbp2-209; fbp4-23; fbp5-51; pm12-37; pm12-118; pm12-325; euap1-317; fbp29-31; fbp29-123 and fbp29-153 were identified by BLAST-searching our sequence-indexed dTph1 transposon flanking sequence database (Vandenbussche et al., 2008) that was
enlarged with the addition of extra populations. Exact insert positions were determined by
aligning the transposon flanking sequences with the corresponding genomic and coding
sequences. The insertion alleles were named after their exact insert position, expressed in bp
downstream of the ATG in the coding sequence (Figures 1G and 5D). Offspring of candidate
insertion lines were grown and genotyped by PCR using gene specific primer pairs flanking the
insertion site (Supplemental Table 2). The following thermal profile was used for segregation
analysis PCR: 11 cycles (94°C for 15s, 71°C for 20s minus 1°C/cycle, 72°C for 30s), followed
by 40 cycles (94°C for 15s, 60°C for 20s, 72°C for 30s). For all alleles, homozygous mutants
were obtained in offspring of the originally heterozygous insertion mutants, either containing
the original transposon insertion allele, or a stably inherited out-of-frame derived footprint
allele that was confirmed by sequencing, fully maintaining the mutation. Insertion alleles that
were used in crosses for higher order mutant analysis are indicated in red in Figures 1G and
5D. The different insertion alleles were further systematically genotyped in subsequent crosses
and segregation analyses. To test genetic interactions with the rob mutations (Figure 6), a pfg
fbp26 fbp29 euap1 mutant was crossed with the earlier described rob1 rob2 rob3 mutant (Morel
et al., 2017). Phenotypic analysis of all single and higher order mutants was focused and limited
to the screening for defects in floral organ development and inflorescence architecture.

Phylogenetic Analysis
The phylogenetic analyses shown in Figures 1F and 5A were conducted using the advanced
PhyML/oneClick workflow at ngphylogeny.fr (Lemoine et al., 2019). Full-length protein
sequences of either SEP/AGL6 (Figure 1F) or AP1/SQUA (Figure 5A) subfamily members
from petunia, tomato, arabidopsis and rice (Supplemental Table 1) were first aligned using
MAFFT (Katoh and Standley, 2013) applying the following options: Data type: Autodetection;
MAFFT flavor: auto; Gap extend penalty: 0.123; Gap opening penalty: 1.53; Matrix selection:
no matrix; Reorder output? true. Output format: FASTA (Supplemental Data files 1 and 2).
Next, alignment curation was done using BMGE (Criscuolo and Gribaldo, 2010) with the
following options: Sequence coding: AA; matrix: BLOSUM; Estimated matrix BLOSUM: 62;
Sliding windows size: 3; Maximum entropy threshold: 0.5; Gap Rate cut-off: 0.5; Minimum
Maximum Likelihood trees were calculated using PhyML (Lemoine et al., 2018) with the
following settings: Data type: amino acids; Evolutionary model: LG; Equilibrium frequencies:
ML/Model. Proportion of invariant sites: estimated; Number of categories for the discrete
gamma model: 4; Parameter of the gamma model: estimated; Tree topology search: Best of
NNI and SPR. Optimize parameter: Tree topology, Branch length, Model parameter; Statistical
test for branch support: Bootstrap; Number of bootstrap replicates: 1000. Seed value used to
initiate the random number generator: 123456. The tree was rendered using Newick Display
(Junier and Zdobnov, 2010). For the visual representation of the SEP/AGL6 analysis (Figure
1F), mid-point rooting was applied on the node separating SEP and AGL6 subfamilies, while
for the AP1/SQUA analysis (Figure 5A), mid-point rooting was applied on the node separating
rice from eudicot AP1/SQUA proteins.

**Imaging and Microscopy**

Electron microscopy images were obtained as previously described (Vandenbussche et al.,
2009) or by using a HIROX SH-1500 benchtop environmental electron microscope equipped
with a cooled stage. Macroscopic floral phenotypes were imaged by conventional digital
photography using a glass plate as a support and black velvet tissue around 10 cm below the
glass plate in order to generate a clean black background. When needed, backgrounds were
further equalized by removing dust particles and light reflections with Photoshop. Images in
Figures 6H, 6I and 6M were photographed using a Zeiss Imager M2 microscope equipped with
an AxioCam MRc camera (Zeiss).

**RT-qPCR Expression Analysis.**

Total RNA was extracted using the Spectrum Plant Total RNA kit (Sigma Aldrich) and treated
with Turbo DNA-free DNase I (Ambion). RNA was reverse transcribed using RevertAid M-
MuLV reverse transcriptase (Fermentas) according to the manufacturer’s protocol. PCR
reactions were performed in an optical 384-well plate in the QuantStudio™ 6 Flex Real-Time
PCR System (Applied Biosystems), using FastStart Universal SYBR Green Master (Roche), in
a final volume of 10µl, according to the manufacturer’s instructions. Primers (Supplemental
Table 2) were designed using the online Universal ProbeLibrary Assay Design Center (Roche).
Data were analyzed using the QuantStudio™ 6 and 7 Flex Real-Time PCR System Software
v1.0 (Applied Biosystems). Petunia *ACTIN*, *GAPDH*, and *RAN* were used as reference genes.
PCR efficiency ($E$) was estimated from the data obtained from standard curve amplification
using the equation $E = 10^{1/slope}$. Relative expression (R.E.) values on the $y$-axes are the average of nine data points resulting from the technical triplicates of three biological replicates ± sd and normalized to the geometrical average of three $E^{ΔCt}$, where $ΔCt = Ct_{GOI} - Ct_{ACTIN, GAPDH}$ and $RAN$.

The floral bud series (marked floral buds 1–3 in Figures 1F, 4G, 5C and Supplemental Figure 1) are successive developmental stages of complete floral buds harvested from the same inflorescences (Figure 1E). Young bracts were harvested from the node bearing stage 3 flowers, while inflorescence stem tissue was collected from the internode connecting node stage 4 and stage 5 bearing flowers. For each biological replicate, corresponding stages harvested from three inflorescences were pooled. Stage 3 corresponds to flower buds with a diameter of ∼5 mm and from which individual floral organs can be easily dissected by hand. All analyses showing expression in separate floral organ types are from this stage. Biological replicates of the different floral organ types were composed of pooled stage 3 organs harvested from three different flowers each time. Floral buds marked “2” (diameter ∼2.5 mm) and “1” (diameter ∼1.5 mm) are younger stages and were harvested from the next two nodes produced after bud stage 3. In addition to 1.5-mm buds, stage 1 also includes the inflorescence meristem and very young developing floral primordia subtended by bracts, which are attached to the base of the pedicel of the 1.5-mm bud. For the sextuple mutant flower buds analyzed in Figure 4G, developmental stages in relation to wild-type development were deduced based on their position on the inflorescence. Vegetative apices (including very small leaf primordia) were harvested from 3-week-old seedlings by manually removing cotyledons, roots, and developed leaves. Young leaf primordia were isolated from the same 3-week-old seedlings. Each biological replicate of the vegetative apices and young leaf primordia consisted of pooled material harvested from each time 10 seedlings. The root samples were obtained by pooling 10-15 actively growing 2 cm root tips per biological replicate.

**Accession Numbers**

Sequence data for the genes that were functionally analyzed in this article can be found in the GenBank/EMBL libraries under accession numbers FBP2 (M91666.1); FBP5 (AF335235.1); PMADS12 (AY370527.1); FBP9 (AF335236.1); FBP23 (AF335241.1); FBP4 (AF335234.1); Ph-AGL6 (AB031035.1); PFG (AF176782.1); FBP29 (AF335245.1); FBP26 (AF176783.1); Ph-euAPI (MK598839) (see also Supplemental Table 1).
Supplemental Data

Supplemental Figure 1. RT-qPCR Expression Analysis of the Petunia UNSHAVEN (UNS) Gene in WT.

Supplemental Figure 2. Further Characterization of the Petunia AP1/SQUA family.

Supplemental Table 1. Gene Names, Synonyms and Accession Codes/Gene Models for Sequences Shown in Figures 1 and 5, in Supplemental Figures 2, and in Supplemental Data Files 1, 2, 3, and 4.

Supplemental Table 2. Oligo Sequences Used in this Study.

Supplemental Data File 1: MAFFT Multiple Alignment of SEP and AGL6 Protein Sequences from Petunia (Ph), Tomato (Sl), Arabidopsis (At) and Rice (Os) in fasta format.

Supplemental Data File 2: MAFFT Multiple Alignment of AP1/SQUA Protein Sequences from Petunia (Ph), Tomato (Sl), Arabidopsis (At) and Rice (Os) in fasta format.


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Author Contributions

M.V. and P.M. conceived and designed the experiments. P.M., P.C., V.B. S.C., F.R., S.R.B., C.T., J.Z., and M.V. performed the experiments. P.M. and M.V. analyzed the data. M.V., M.M. and P.M. wrote the manuscript.

REFERENCES


in the transition from vegetative to reproductive development. Development 126, 5117-5126.


FIGURE LEGENDS

Figure 1. Characterization of the Petunia SEP/AGL6 MADS-box Genes.

(A) Section through a WT petunia W138 flower showing inner whorls. (B) Petunia seedpod ~4 weeks post-pollination surrounded by green sepals. (C) SEM (scanning electron microscopy) images of sepal, bract and leaf adaxial and abaxial epidermal surfaces. Bars = 50 µm. (D) Longitudinal sections of developing petunia floral buds showing the placenta developing from the center of the floral meristem s = sepal; p = petal; st = stamen; c = carpel; pl = placenta. Bars = 200 µm. (E) W138 floral bud developmental stages for RT-qPCR analysis shown in (G), dissected from the top of an inflorescence (inset), of which the large floral bud at the right is just prior to opening. Numbers indicate sampled stages. 1 to 3 correspond to floral bud diameters of ~1.5, 2.5 and 5 mm respectively. Stage 1 also includes very early flower primordia, bracts and the inflorescence meristem. Bar = 1 cm. (F) Maximum Likelihood phylogenetic analysis of the SEP and AGL6 subfamily members of Petunia hybrida (Ph), Solanum lycopersicum (Sl), Arabidopsis thaliana (At) and Oryza sativa (Os). Bootstrap values marked in red (expressed in %, based on 1000 replicates) supporting tree branching are indicated near
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tissues (green bars; infl. stem = inflorescence stem); entire floral buds (orange bars) from 3
developmental stages shown in (E) and dissected floral organs (red bars) obtained from flower
buds corresponding to stage 3. (H) Schematic representations of the gene structures and
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respectively. All gene models start at the start codon and end at the stop codon. Scale bars =
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creation of double and higher order mutants are marked in red.

**Figure 2. The Petunia fbp2 fbp5 pm12 Mutant, Genetic Equivalent of the Arabidopsis sep1 sep2 sep3 Mutant, Still Displays B- and C-function Floral Characteristics.**

(A) to (H) Top view of flowers from WT, single, double and triple mutants of petunia
SEP1/SEP2/SEP3 homologs. All images are at the same magnification. (I) to (L) Side view of
WT and mutant flowers sectioned through the middle. All images are at the same magnification.
(M) Close-up of dissected third whorl organs (stamens). (N) Close-up of dissected fourth whorl
organs (carpels). (O) to (Q) SEM images of the outer ovary surface. Scale bars = 100 µm.

**Figure 3. Petunia Floral Meristem Identity Depends on FBP9/FPB23/FPB4 Activity.**

(A) to (C) and (E) to (G) Top and side view of WT, fbp9 fbp23 and fbp9 fbp23 fbp4 plants 13
weeks after sowing. (D) and (H) Schematic representation of inflorescence phenotypes. (I) to
(L) SEM images of inflorescence apices in WT and fbp4 fbp9 fbp23 mutants. Br: bracts; Se:
sepals; F: flower; Fm: Flower meristem; Im: Inflorescence meristem. Scale bars = 100 µm. (M)
to (Q) Inflorescence architecture of lower order mutants compared to WT and fbp9 fbp23 fbp4
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(A) and (B) Top view of young (A) and mature flower (B). (C) Dissected floral organs of a flower similar to the stage as indicated by the asterisk in (F). W# indicate whorl numbers. (D) Longitudinal section through an older flower similar to the stage as indicated by the double asterisk in (F). (E) SEM images of the epidermis of the four different floral whorls (indicated by W#) in WT (left panels) and the sextuple mutant (right panels). (F) Inflorescences showing flowers at various stages of development and aging. The arrows indicate an example where three consecutive fully developed flowers arose from a single floral meristem. Scale bars: 0.25 cm in (A); 0.5 cm in (B, D); 1 cm in (C, F); 50 µm in (E). (G) RT-qPCR expression analysis of the petunia floral homeotic genes in WT versus sextuple \textit{fbp2 fbp4 fbp5 fbp9 pm12 agl6} mutants. Petunia genes are indicated and names of corresponding Arabidopsis orthologs are shown in between brackets. *No TM6 ortholog exists in the Arabidopsis genome. **Petunia \textit{FBP6} is orthologous to \textit{SHP1}/\textit{SHP2}, but is functionally homologous to \textit{AG}. Relative expression (R.E.) levels are plotted as the mean value of three biological and three technical replicates ±SE, normalized against three reference genes (see Material and Methods). Expression levels were measured in entire floral buds from three developmental stages as shown in Figure 1E.

Figure 5. Characterization of the Petunia AP1/SQUA MADS-box Subfamily. 
(A) Maximum likelihood phylogenetic analysis of the AP1/SQUA subfamily members of \textit{Petunia hybrida} (Ph), \textit{Solanum lycopersicum} (Sl), \textit{Arabidopsis thaliana} (At) and \textit{Oryza sativa} (Os). Bootstrap values marked in red (expressed in %, based on 1000 replicates) supporting branching are indicated near the branch points. The scale bar represents number of substitutions/site. Accession codes for the corresponding sequences are shown in Supplemental Table 1. Naming of subfamilies and subfamily clades is based on previously described phylogenies for the AP1/SQUA subfamily (Litt and Irish, 2003; Yu et al., 2016; Maheepala et al., 2019). (B) RT-qPCR expression analysis of the petunia \textit{AP1/SQUA} genes. Relative expression (R.E.) levels are plotted as the mean value of three biological and three technical replicates ±SE, normalized against three reference genes (see Material and Methods). See legend of Figure 1G for sample description. (C) Schematic representations of the gene structures and insertion alleles of the petunia \textit{AP1/SQUA} genes and corresponding floral
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(A) to (D) Flower phenotype of *pfg fbp26 fbp29 euap1* mutants. Some sepals and petals have been removed in (B) to reveal inner organs. (D) Enlarged sepals showing petaloid sectors displaying petal conical epidermal cells (inset SEM image). (E) to (G) Inflorescence phenotype showing an “inflorescence” with spirally organized leaves (F) ending in a single terminal flower (G). (L) Side branches developing from the basis of the plant exhibit an identical inflorescence phenotype. (H), (I) and (M) Longitudinal sections of the apex of an inflorescence in vegetative state (G), and of an inflorescence with terminal flower (I), compared to the apex of a WT inflorescence (M). Red asterisks in (I) indicate vegetative lateral meristems. (J) to (K) Enhanced homeotic sepal-to-petal conversion compared to (C) and (D). (N) and (O) unmodified sepals in *rob1 rob2/+ rob3* mutants (N) compared to WT (O). (P) and (Q) SEM images of a WT vegetative meristem before the onset to flowering compared to the apex of a *pfg fbp26 fbp29 euap1* inflorescence prior to terminal flower formation as in (H). (R) Schematic representation of a *pfg fbp26 fbp29 euap1* inflorescence (right) compared to an intermediate inflorescence phenotype as shown in (T) to (V). (S) to (X) Inflorescence phenotypes of WT, quadruple and various triple mutant combinations after prolonged flowering. White arrows indicate positions of previous terminal flowers. Scale bars: 1 cm in (A-G; J-L; N-O; S-X); 100 µm in (P-Q; H, I, M); 50 µm in inset in (D).
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