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► **To cite this version:**

Patrice Morel, Pierre Chambrier, Véronique Boltz, Sophy Chamot, Frédérique Rozier, et al.. Divergent Functional Diversification Patterns in the SEP/AGL6/AP1 MADS-box Transcription Factor Superclade. *The Plant cell*, 2019, tpc.00162.2019. 10.1105/tpc.19.00162 . hal-02349131

HAL Id: hal-02349131

<https://hal.science/hal-02349131>

Submitted on 5 Nov 2019

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1 **RESEARCH ARTICLE**

2
3 **Divergent Functional Diversification Patterns in the SEP/AGL6/API**
4 **MADS-box Transcription Factor Superclade**

5
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14
15 **Short title:** Analysis of the Petunia SEP/AGL6/API Superclade

16
17 **One sentence summary:** Functional analysis of the petunia MADS-box gene SEP/AGL6/API
18 superclade compared to Arabidopsis and other species suggests major differences in the functional
19 diversification of its members during evolution.

20
21 **Keywords:** SEPALLATA; APETALA1; API/SQUA; AGL6; MADS-box; floral meristem identity;
22 inflorescence meristem identity, plant evolution; ABC model; Petunia; Arabidopsis; inflorescence
23 architecture

24
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26 accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Michiel
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28
29 **ABSTRACT**

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

44

45 INTRODUCTION

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

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

75 Thus far, *Arabidopsis* is the only core eudicot species for which a functional
76 characterization of all its *API/SEP/AGL6* superclade genes has been achieved in sufficient
77 detail, including the identification of redundant functions through higher order mutant analysis,
78 but a wealth of functional data has been accumulated also in tomato and rice in recent years

79 (see further). The Arabidopsis *SEP* subfamily consists of four members, named *SEP1*, *SEP2*,
80 *SEP3* and *SEP4*, and petals, stamens and carpels in the *sep1 sep2 sep3* triple mutant are
81 transformed into sepals (Pelaz et al., 2000), while all floral organs in a *sep1 sep2 sep3 sep4*
82 mutant develop as leaf-like organs (Ditta et al., 2004). This led to the conclusion that *SEP* genes
83 are required for the identity of all floral organs, and function in a largely, but not completely
84 redundant fashion. In addition, *SEP* genes were shown to be involved in floral meristem identity
85 and determinacy (Pelaz et al., 2000; Ditta et al., 2004). *SEP* proteins are proposed to act as
86 ‘bridge proteins’ enabling higher order complex formation (floral quartets) with the products
87 of the homeotic B and C function organ identity genes, and to provide transcriptional activation
88 capacity to these complexes (Honma and Goto, 2001; Theissen and Saedler, 2001; Immink et
89 al., 2009; Melzer et al., 2009). These findings have inspired the addition of the *SEP* (or *E-*)
90 function to the classic ABC model of floral development (Bowman et al., 1991; Coen and
91 Meyerowitz, 1991), summarized in a floral quartet model (Theissen and Saedler, 2001). In
92 contrast to the function of *AGL6* genes in other species, the two Arabidopsis *AGL6* subfamily
93 members *AGL6* and *AGL13* do not seem to perform a *SEP*-like function in floral organ identity
94 determination (Koo et al., 2010; Huang et al., 2012; Hsu et al., 2014). The Arabidopsis
95 *API/SQUA* subfamily is composed of 4 members, of which the roles of *API*, *CAL*
96 (*CAULIFLOWER*) and *FUL* (*FRUITFULL*) in floral development have been particularly well
97 studied. Arabidopsis *apl* mutants lack petals and have sepals displaying bract like features
98 (Irish and Sussex, 1990; Mandel et al., 1992). For these reasons, *API* has been classified as an
99 A-function gene in the ABC model, required for the identity specification of sepals and petals.
100 Furthermore, *API* plays also a major role in specifying floral meristem identity, in a largely
101 redundant fashion with *CAL* (Bowman et al., 1993; Kempin et al., 1995). *FUL* was initially
102 identified for its unique role in Arabidopsis carpel and fruit development (Gu et al., 1998), but
103 in addition was later shown to function redundantly with *API* and *CAL* to control inflorescence
104 architecture (Ferrandiz et al., 2000).

105 To provide more insight in floral development and in the evolution of the floral gene
106 regulatory network in higher eudicot species in general, we have been systematically analyzing
107 the genetics underlying floral development in the Asterid species *Petunia x hybrida*. While the
108 genes encoding the floral A, B and C- functions in petunia have been well characterized
109 (Angenent et al., 1993; van der Krol et al., 1993; Kater et al., 1998; Kapoor et al., 2002;
110 Vandenbussche et al., 2004; Rijpkema et al., 2006; Cartolano et al., 2007; Heijmans et al., 2012;
111 Morel et al., 2017; Morel et al., 2018), only a few of the 10 previously described genes
112 belonging to the large petunia *API/SEP/AGL6* superclade (Immink et al., 1999; Ferrario et al.,

113 2003; Immink et al., 2003; Vandenbussche et al., 2003a; Vandenbussche et al., 2003b;
114 Rijpkema et al., 2009) have been functionally analyzed thus far.

115 Research on petunia *SEPALLATA* genes dates back a long time and provided, together
116 with a study in tomato, the first indication of the existence of SEP-function in floral
117 development: transgenic lines in which the *SEP3*-like petunia *FBP2* or tomato *TM5* genes were
118 silenced by co-suppression both exhibited simultaneous homeotic conversion of whorls 2, 3,
119 and 4 into sepal-like organs and loss of determinacy in the center of the flower (Angenent et
120 al., 1994; Pnueli et al., 1994), a phenotype similar to that later found in Arabidopsis *sep1 sep2*
121 *sep3* mutants. However, at that time, multimeric complex formation of MADS-box proteins
122 still remained to be discovered (Egea-Cortines et al., 1999), and it was not clear how many
123 genes were co-suppressed in these lines. Therefore, the molecular basis of these phenotypes
124 in petunia and tomato was not immediately understood. Later, it was shown in yeast that petunia
125 SEP proteins also bind to B-class heterodimers and to C-class proteins, mediate quaternary
126 complex formation with B- and C-class proteins and display transcriptional activation capacity
127 (Ferrario et al., 2003), compatible with the proposed quartet model in Arabidopsis.
128 Interestingly, among the six petunia SEP-like proteins, also clear differences in protein–protein
129 interactions were revealed in a yeast 2-hybrid assay, suggesting functional diversification
130 (Ferrario et al., 2003; Immink et al., 2003). Especially *FBP2* and *FBP5* showed a much broader
131 range of interaction partners compared to the other petunia SEP proteins. Furthermore, it was
132 shown *in planta* that petunia SEP proteins may be crucial to import at least some other MADS-
133 box transcription factors into the nucleus (Immink et al., 2002).

134 Using a gene-specific approach, we showed that the *fbp2* co-suppression phenotype was
135 indeed not gene specific, since single *fbp2* mutants showed only a very incomplete *sep-like*
136 phenotype, with primarily the margins of the petals exhibiting a petal-to sepal homeotic
137 conversion (Vandenbussche et al., 2003b). We also reported *fbp5* mutants that as single mutants
138 develop as wild type. Flowers of *fbp2 fbp5* mutants, however, showed an enhanced phenotype
139 compared to *fbp2* mutants: the sepaloid regions at the petal edges extended slightly further
140 towards the center; sepal-like structures appeared on top of the anthers, and a sudden dramatic
141 phenotype appeared in the ovary, which continued to grow long after development has arrested
142 in wild-type (WT) flowers of comparable stages, resulting in a giant ovary. While the general
143 architecture of the ovary was maintained (carpels containing an interior placenta), inside all
144 ovules were homeotically converted to sepal-like organs (Vandenbussche et al., 2003b). This
145 directly demonstrated that not only the identity of petals, stamens and carpels depends on SEP
146 activity in petunia, but also ovule identity, as was also reported in Arabidopsis in the same

147 journal issue (Favaro et al., 2003). More recently, we demonstrated that petunia *AGL6* also
148 exhibits SEP-like functions (Rijkema et al., 2009), and performs a major role in petal identity,
149 redundantly with *FBP2*. In addition, a function in stamen development was revealed by *fbp2*
150 *fbp5 agl6* triple mutant analysis. In line with the proposed SEP-function for *AGL6*, we found
151 that *AGL6* and *FBP2* in yeast overall interact with the same the partners (Rijkema et al., 2009).

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

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

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

176 Finally, we show that the petunia *API/SQUA* subfamily is composed of four members
177 (*PFG*, *FBP26*, *FBP26* and the here described *euAPI* gene) that function in a largely redundant
178 way. We found that they are required for inflorescence meristem identity, but surprisingly, *pfg*
179 *fbp26 fbp29 euap1* mutants developed fully functional terminal flowers. In addition, we show
180 that they act as B-function repressors in the first floral whorl, together with *BEN/ROB* genes

181 (Morel et al., 2017). Overall, comparison of these data with previous studies in mainly
182 Arabidopsis, tomato and rice reveal major differences in the functional diversification of the
183 *SEP/AGL6* and *API/SQUA* MADS-box subfamilies during evolution of the angiosperms.

184

185 **RESULTS**

186 **Petunia Floral Development**

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

212

213 **Petunia *SEP/AGL6* Expression Analysis and Mutant Identification**

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

230 As expected based on their close taxonomic relationship, the petunia proteins overall
231 showed the closest relationship with *SEP/AGL6* members from tomato (The Tomato Genome
232 Consortium, 2012; Soyk et al., 2017) compared to Arabidopsis and rice (Figure 1F). Like
233 petunia, tomato contained one *AGL6* gene, one *SEP3* copy and two *FBP9* members, but slight
234 differences in the number of genes belonging to the *SEP1/SEP2* and *SEP4* subclades could be
235 observed between the two species. Notably, tomato contained only one *SEP1/SEP2* copy, while
236 having two *SEP4*-like genes. Among the members of the tomato *SEP/AGL6* family, the *SEP4*-
237 like *RIN* gene initially received most of the attention, because the classical *rin* mutation has
238 been widely used in tomato breeding as it improves shelf-life of tomato fruits when present in
239 a heterozygous state, while the homozygous *rin* mutation prevents initiation of ripening
240 (Vrebalov et al., 2002). Interestingly, more recent studies in tomato have shed a first light on
241 the function of the enigmatic *FBP9* subclade genes. First of all, it was found that *SLMBP21/J2*
242 (*JOINTLESS 2*) is required for the development of the pedicel abscission zone (Liu et al., 2014;
243 Roldan et al., 2017; Soyk et al., 2017). Furthermore, a breakthrough functional study (Soyk et
244 al., 2017) based on both natural and CRISPR induced mutant alleles showed that the two tomato
245 *FBP9* clade genes *SLMBP21/J2* and *SIMADS1/EJ2* (*ENHANCER OF JOINTLESS 2*) have
246 overlapping functions in meristem maturation and the control of inflorescence branching
247 together with *LIN* (*LONG INFLORESCENCE*), the second tomato *SEP4*-like gene.

248 Remarkably, triple *j2 ej2 lin* knockout mutants exhibit a dramatic phenotype consisting of
249 massively overproliferated sympodial inflorescence meristems (SIMs) without the formation
250 of flowers, indicating that the transition towards FM identity is not made.

251 As a first step in the characterization of the complete *SEP/AGL6* clade in petunia, we
252 performed RT-qPCR expression analysis (Figure 1G) in three floral bud developmental stages
253 (Figure 1E) with the stage 1 floral bud sample also including very early flower primordia, bracts
254 and the inflorescence meristem, and in various other tissues. This allows for a more quantitative
255 analysis than a previous study by RNA gel blot and *in situ* hybridization (Ferrario et al., 2003).
256 We detected important differences in expression levels among the *SEP/AGL6* genes and clear
257 differences in expression patterns, both correlated with their phylogenetic position, suggesting
258 functional divergence: *FBP2*, *FBP5*, and *AGL6* were the most abundantly expressed genes,
259 reaching expression levels roughly tenfold higher than the *SEP4* homolog *FBP4*, and the *FBP9*
260 subclade members *FBP9* and *FBP23*. Furthermore, *FBP2*, *FBP5* and *AGL6* expression was
261 restricted to floral tissues, with expression levels strongly increasing during floral bud
262 development, while *FBP4*, *FBP9* and *FBP23* were more broadly expressed, and expression
263 levels did not show a strong upregulation during later stages of floral bud development.
264 Expression outside the floral domain was most marked in bracts for *FBP4*, and in the
265 inflorescence stem tissue for both *FBP4* and *FBP9*. One exception to these general differences
266 observed between *SEP1/SEP2/SEP3/AGL6* and *SEP4/FBP9* genes was *PM12*, which was
267 expressed ~100 fold lower than its close paralog *FBP5*, and for which expression was detected
268 also in bracts and stems. For all genes analyzed, expression levels varied considerably between
269 the different floral organs: Expression may be much lower in one particular organ type
270 compared to the three other floral organs (e.g. very low *FBP2* and *FBP5* expression in sepals;
271 low levels of *PMADS12*, *AGL6* and *FBP23* expression in stamens), or may peak in one specific
272 floral organ (*FBP4* in sepals; *FBP9* in petals). Our results are in agreement with the *in situ* data
273 previously obtained for *FBP2* and *FBP5*, showing mainly expression in the three inner floral
274 whorls during early flower development, while some minor differences with the *PMADS12 in*
275 *situ* data suggest that the *PM12* expression pattern is not constant as floral buds further develop
276 (Ferrario et al., 2003).

277 To perform a functional analysis, we used a reverse genetics strategy (Koes et al., 1995;
278 Vandenbussche et al., 2003b; Vandenbussche et al., 2008) to identify *dTph1* transposon
279 insertions in the coding sequences of the petunia *SEP* and *AGL6* genes. In total, we identified
280 and confirmed 16 independent transposon insertions *in planta*, including some earlier reported
281 alleles (Vandenbussche et al., 2003b; Rijpkema et al., 2009) in all of the 7 different members

282 of the *SEP/AGL6* clade (Figure 1H). Because the 284 bp *dTph1* sequence encodes stop codons
283 in all six possible reading frames, and based on their insert position (either disrupting the first
284 exon encoding the MADS DNA binding domain, or the K-region required for protein-protein
285 interactions in the case of the *fbp2* insertions, all of the selected insertion alleles most likely
286 represent null alleles. We obtained and analyzed homozygous mutants for all insertion alleles,
287 but remarkably, only homozygous mutants for *fbp2* insertions displayed floral homeotic defects
288 (Figure 1H), suggesting extensive functional redundancy among the petunia *SEP/AGL6* genes,
289 and that *FBP2* function is more essential than that of any other *SEP/AGL6* gene. These results
290 clearly indicated the need for multiple mutant analyses to further uncover putative redundant
291 functions.

292

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

296 In Arabidopsis, the simultaneous loss of *SEP1*, *SEP2* and *SEP3* results in flowers consisting
297 only of sepals (Pelaz et al., 2000). To compare the petunia genetic equivalent, we aimed to
298 analyze *fbp2 fbp5 pm12* triple mutants (Figure 2). As mentioned earlier, of the three single
299 mutants, only *fbp2* mutants displayed a phenotype different from WT (Figures 2A to 2D).
300 Moreover, *fbp2/+ fbp5 pm12* flowers (Figure 2E) developed morphologically as WT,
301 demonstrating that *FBP2* even in a heterozygous state can fully compensate for the loss of
302 *FBP5* and *PM12* functions. In addition, *fbp2 pm12* double mutants were not markedly different
303 from *fbp2* single mutants (Figure 2F), in contrast to the earlier reported *fbp2 fbp5* mutants
304 (Figures 2G to 2K) and (Vandenbussche et al., 2003b). However, *fbp2 fbp5 pm12* flowers could
305 be easily distinguished from *fbp2 fbp5* flowers: a clear enhancement of stamen to sepal identity
306 could be observed in the third whorl, although still some antheroid tissue remained, as in *fbp2*
307 *fbp5* mutants (Figure 2M). Furthermore, while the extremely enlarged *fbp2 fbp5* mutant pistil
308 still exhibited partial carpel identity, the carpels of *fbp2 fbp5 pm12* mutants acquired clear
309 sepal/leaf-like epidermal characteristics (Figures 2N to 2Q), and were densely covered with
310 trichomes. The latter are never observed on WT pistils, and only at very low frequency on *fbp2*
311 *fbp5* pistils (Figure 2N). Furthermore, no stigma and style structures remained in the triple
312 mutant, but the overall internal organization of the ovary was maintained, with a placenta
313 structure covered by a few hundred leaf-like organs that represented homeotically converted
314 ovules, as observed in *fbp2 fbp5* mutants (Figures 2K and 2L). In the second whorl of *fbp2 fbp5*
315 *pm12* flowers, the partial petal to sepal conversion at the corolla border was only subtly

316 enhanced compared to *fbp2 fbp5* mutants (Figures 2G and 2H). Given that the effect of the
317 *pm12* mutation only became apparent in an *fbp2 fbp5* mutant background, we conclude that
318 *PM12* plays a less essential role than its close paralog *FBP5*. Overall, the remnant petal and
319 stamen tissues and the maintenance of a placenta structure in *fbp2 fbp5 pm12* flowers show that
320 unlike in Arabidopsis, genes outside the *SEP3* and *SEP1/SEP2* subfamilies are able to rescue
321 part of the B- and C-functions in a petunia *sep1 sep2 sep3* mutant background.

322

323 **The *FBP9* Subclade Genes *FBP9* and *FBP23* Function as Floral Meristem Identity Genes** 324 **together with *FBP4***

325 We showed earlier that petunia *AGL6* is one of the genes outside the classical *SEP1/SEP2/SEP3*
326 group that plays a prominent role in performing a *SEP*-like floral organ identity function,
327 especially in the determination of petal identity, redundantly with *FBP2* (Rijkema et al., 2009).
328 However, *FBP4* as a *SEP4*-like gene may also participate, as found in Arabidopsis (Ditta et al.,
329 2004) and potentially also *FBP9* and *FBP23*, the petunia representatives of the *FBP9* subclade.

330 Earlier we found that the *fbp9*, *fbp23* and *fbp4* single mutants displayed a WT phenotype
331 (Figure 1H), and that expression levels of all three genes peak early during floral developmental
332 stages compared to the other petunia *SEP* genes and *AGL6* (Figure 1G), potentially indicating
333 a redundant (common) function for *FBP4*, *FBP9* and *FBP23*. Indeed, a functional overlap was
334 recently demonstrated among corresponding *SEP* subclade members in tomato (Soyk et al.,
335 2017).

336 To test such a putative functional redundancy among the petunia *FBP9*, *FBP23* and
337 *FBP4* genes, we first created and analyzed *fbp9 fbp23* double mutants, since *FBP9* and *FBP23*
338 are close paralogs belonging to the same *FBP9 SEP*-subclade. Interestingly, we found that *fbp9*
339 *fbp23* mutants were dramatically affected in their inflorescence architecture, with new
340 inflorescence shoots developing instead of flowers, resulting in a highly branched inflorescence
341 structure. However, flower development was not completely abolished in these mutants,
342 because after several weeks of a highly branched inflorescence development, frequently a
343 flower appeared on one or more branches of the same plant, after which these branches switched
344 again to the initial phenotype (Figures 3B and 3F). This indicated that the capacity to form
345 floral meristems was not completely abolished in *fbp9 fbp23* mutants and that (an)other
346 factor(s) can partly rescue floral meristem determinacy in the absence of *FBP9/FBP23* function.
347 Because we assumed *FBP4* being a likely candidate, we next analyzed *fbp4 fbp9 fbp23* triple
348 mutants. Indeed, we found that the *fbp9 fbp23* phenotype was further enhanced in these triple
349 mutants, resulting in a highly branched flowerless inflorescence architecture (Figures 3C, 3G

350 and 3H), phenotypically very similar to that reported earlier for the petunia floral meristem
351 identity mutant *alf* (Souer et al., 1998), with *ALF* being orthologous to Arabidopsis *LEAFY*
352 (*LFY*) (Weigel et al., 1992) and snapdragon *FLORICAULA* (*FLO*) (Coen et al., 1990) genes.
353 Note that over a long period (> 6 months) of highly branched inflorescence development, some
354 triple mutants produced 1-2 isolated flowers, while other individuals never flowered at all.

355 To study the *fbp4 fbp9 fbp23* phenotype in more detail, we analyzed *fbp4 fbp9 fbp23*
356 inflorescence apices by scanning electron microscopy in comparison with WT (Figures 3I to
357 3L). At very early developmental stages, the *fbp4 fbp9 fbp23* plants exhibited a phenotype very
358 comparable to *alf* mutants (Souer et al., 1998): as in *alf* mutants, the bifurcation pattern of *fbp4*
359 *fbp9 fbp23* inflorescence apices was similar to WT, but the two resulting meristems both
360 behaved as inflorescence meristems, as indicated by the continuous bifurcation of each newly
361 formed meristem and the repetitive formation of bracts flanking these meristems. Together, this
362 indicates that floral meristems in *fbp4 fbp9 fbp23* mutants are homeotically transformed into
363 inflorescence meristems.

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

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

377 To test whether *FBP4*, *FBP9* and *FBP23* also function later in conferring floral organ
378 identity, we introduced the corresponding mutations into the *fbp2* mutant background, the only
379 petunia *sep* mutation with a visible phenotype as a single mutant. However, we found that
380 flowers of *fbp2 fbp4*, *fbp2 fbp9* and *fbp2 fbp23* mutants were not markedly different from *fbp2*
381 mutants (Figures 3R to 3U), while *fbp2 fbp4 fbp23* and *fbp2 fbp4 fbp9* flowers only showed a
382 moderate enhancement of the *fbp2* petal-to-sepal conversion phenotype (Figure Figures 3V to
383 3W). In *fbp2 fbp4 fbp23* flowers, the green margin appeared to be broader in all five petals

384 while in *fbp2 fbp4 fbp9* flowers this was most visible in the two basal petals. In comparison
385 with the earlier described floral phenotypes of *fbp2 fbp5* (Vandenbussche et al., 2003b), *fbp2*
386 *agl6*, *fbp2 fbp5 agl6* (Rijpkema et al., 2009) and *fbp2 fbp5 pmads12* mutants, this suggests that
387 *FBP4*, *FBP9* and *FBP23* do play a role in floral organ identity, but contribute only moderately
388 to this function compared to the petunia *SEP1/SEP2/SEP3* homologs and *AGL6*. Note that we
389 also obtained *fbp2 fbp4 fbp9 fbp23* quadruple mutants, but as expected, these developed a
390 highly branched flowerless inflorescence structure as in *fbp4 fbp9 fbp23* mutants.

391

392 **The Sextuple *fbp2 fbp4 fbp5 fbp9 pm12 agl6* Mutant Displays a Classic *sepallata* Phenotype**

393 By analyzing *AGL6*, *FBP2* and *FBP5* functions and the *fbp2 fbp5 pmads12* and *fbp4*
394 *fbp9 fbp23* triple and *fbp2 fbp4 fbp9 fbp23* mutants, we could reveal specific/specialized SEP
395 functions for certain members of the petunia *SEP/AGL6* clade and surprisingly, the requirement
396 of *FBP9/FBP23* function (and to a lesser extend *FBP4*) for floral meristem identity, as was also
397 recently shown in tomato. However, a classic floral *sepallata* phenotype as described for
398 Arabidopsis was not obtained, indicating further redundancy, possibly shared between the
399 majority of the petunia *SEP/AGL6* genes. To test this further, we embarked on a long-term
400 crossing scheme aimed to combine all of the *sep/agl6* mutant alleles in a single plant. However,
401 we chose to exclude the *fbp23* mutation in this scheme since this would completely abolish
402 flower formation when combined with the *fbp9* and *fbp4* mutations and thus prevent
403 visualization of additive floral phenotypes. After years of crossing, we finally obtained
404 homozygous sextuple *fbp2-332 fbp4-44 fbp5-129 fbp9-90 pm12-37 agl6-118* mutant plants,
405 hereafter referred to as sextuple *sep/agl6* mutants. In contrast to the earlier described lower
406 order mutants, all organs in the flowers of sextuple *sep/agl6* mutants were green and densely
407 covered by trichomes (Figure 4), exhibiting sepal/leaf-like characteristics (Figures 4A to 4E).
408 Note that as mentioned earlier, it is not possible to discriminate between sepal, bract and leaf
409 identity in petunia based on epidermal cell characteristics (Figure 1C). As expected, scanning
410 electron microscopy of these organs revealed the conversion of the typical petal, stamen and
411 carpel epidermal cell types into epidermal cells characteristic for sepals, bracts and leaves,
412 including stomata and multicellular trichomes (Figure 4E). The second whorl, which in WT
413 consists of five large brightly colored fused petals, was occupied by five green organs that
414 remained fused at their bases (Figure 1C). Although dramatically smaller than WT petals, they
415 remained larger than first whorl sepals. Similarly, in the third whorl, the five stamens were
416 replaced by sepal/leaf like organs. The overall shape of these organs did retain some of the
417 stamen architecture, since the region corresponding to the WT stamen filament remained

418 smaller compared to the more leaf blade-like upperparts. Stamen filaments in WT are fused
419 along half of their length with the inside of the petal tube. By contrast, third whorl organs in the
420 sextuple mutant completely lost this partial fusion. In the fourth whorl, normally occupied by
421 two carpels that are entirely fused and enclose the placenta and ovules, two (sometimes three)
422 unfused sepal/leaf-like organs were found. Internally, the placenta was entirely replaced by a
423 new emerging flower reiterating the same floral phenotype (Figure 1D). Thus in contrast to
424 lower order *sep* mutants, the sextuple mutant was fully indeterminate. In the majority of the
425 flowers (Figure 4F), this secondary flower further developed and emerged from the primary
426 flower supported by a pedicel, while containing on itself another flower in its center. This third
427 flower usually did not further grow out, although occasionally we observed up to three
428 consecutive fully developed flowers (Figure 4F). Note that the sextuple *sep/agl6* mutant
429 displayed a normal cymose inflorescence architecture as in WT (Figure 4F), in sharp contrast
430 to *fbp4 fbp9 fbp23* mutants. This demonstrates that *FBP23* alone can fully rescue floral
431 meristem identity in a sextuple mutant background, but not floral organ identity.

432

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

434 To further characterize the sextuple *sep/agl6* mutant at the molecular level, we quantified and
435 compared the dynamics of homeotic gene expression levels between WT and the sextuple
436 mutant (Figure 4G) at three different stages of floral bud development, as described earlier
437 (Figure 1E). Encoding of the B-function in petunia is more complex compared to Arabidopsis
438 and Antirrhinum, and involves the two PI/GLO-like MADS-box transcription factors *Petunia*
439 *hybrida GLO1* and *GLO2*, the DEF/AP3 ortholog *PhDEF*, and *PhTM6*, the petunia
440 representative of the ancestral B-class TM6 lineage that has been lost in Arabidopsis, but which
441 is present in many species (Angenent et al., 1993; van der Krol et al., 1993; Vandenbussche et
442 al., 2004; Rijpkema et al., 2006). In WT, we found that all four B-class genes were
443 progressively upregulated as floral buds developed, with an upregulation from stage 1 to stage
444 3 varying roughly from three to six times, depending on the gene. In the sextuple mutant, we
445 observed expression levels of *PhGLO1*, *PhGLO2* and *PhDEF* initially similar to WT in the
446 youngest stage analyzed. However, upregulation in older stages was strongly affected,
447 especially for *PhGLO1* and *PhGLO2*, which remained expressed at initial levels. *PhDEF*
448 remained progressively upregulated in the different sextuple mutant samples, but reached only
449 one third of the expression compared to WT in the final stage. By contrast, *PhTM6* expression
450 levels were strongly downregulated from stage 1 floral buds onwards, remaining at similarly
451 low levels in the two older stages.

452 The C-function in petunia is redundantly encoded by *PMADS3* and *FBP6*, orthologs of
453 Arabidopsis AG and SHP1/2 respectively (Heijmans et al., 2012; Morel et al., 2018). As for the
454 B-function genes, *FBP6* and *PMADS3* expression in WT is progressively upregulated in
455 developing floral buds (6,5 and 11 times respectively), and initial expression levels in stage 1
456 buds were very similar between WT and sextuple mutants for both C-class genes. Both C-genes
457 still displayed a clear upregulation in the older sextuple mutant flower buds, and seemed in
458 general less affected by the sextuple loss of SEP/AGL6 function than the B-function genes,
459 especially in stage 2. In stage 3 buds, *FBP6* expression was not different between WT and
460 sextuple mutants, while *PMADS3* in the sextuple mutant was expressed at around 50% of its
461 WT levels. *FBP11* is a petunia D-lineage MADS-box gene orthologous to *STK* (Angenent et
462 al., 1995; Colombo et al., 1995), and that with *FBP7* (another D-lineage member) and the C-
463 genes *PMADS3* and *FBP6* redundantly is required to confer ovule identity, and to arrest the
464 floral meristem (Heijmans et al., 2012). Consistent with its later function in floral development,
465 the *FBP11* expression profile showed a very strong upregulation in the WT developmental
466 series (~30 fold). By contrast, in the sextuple mutant samples, *FBP11* expression was barely
467 detectable in all stages tested.

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

481

482 **The Petunia *API/SQUA* Subfamily: Phylogeny, Expression Analysis and Mutant** 483 **Identification**

484 We found that the petunia *SEP* genes *FBP9*, *FBP23* and *FBP4* function primarily as
485 floral meristem identity genes, a function which is in Arabidopsis mainly associated with

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

513 The previously described *FBP29* gene fell into the *AGL79* subclade to which the tomato
514 genes *MBP10* and *MBP20* also belonged, while the *PFG* and *FBP26* genes grouped into the
515 *euFUL* subclade together with the tomato *FUL1 (TDR4/TM4)* and *FUL2 (MBP7)* genes as
516 previously shown (Yu et al., 2016). While stable mutants remain to be described for these four
517 tomato genes, RNAi mediated downregulation of *FUL1* and *FUL2* indicate a role for these
518 genes in fleshy fruit ripening (Bemer et al., 2012; Shima et al., 2013; Wang et al., 2014).
519 Furthermore, a role for *MBP20* and *FUL1* was proposed in the regulation of compound leaf

520 development (Burko et al., 2013). Finally, to date no function has been proposed for tomato
521 *MBP10* but an evolutionary study of the *FUL* genes in the Solanaceous family suggest that the
522 *MBP10* lineage, which is absent in petunia, may be undergoing pseudogenization (Maheepala
523 et al., 2019). A sequence analysis of the *Petunia axillaris* and *Petunia inflata* genome sequences
524 (Bombarely et al., 2016) further indicated that *euAPI*, *PFG*, *FBP26* and *FBP29* represent the
525 total number of *API/SQUA* family members in petunia (Supplemental Table 1), similar to the
526 size of the *API/SQUA* subfamily in Arabidopsis and rice, and one less compared to tomato (due
527 to the absence of a *MBP10* lineage member in petunia).

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

545 To determine the function of the four petunia *API/SQUA* genes, we screened for *dTph1*
546 transposon insertions in their coding sequences, similarly as for the *SEP* genes. In total, we
547 identified and confirmed 6 independent transposon insertions *in planta* (Figure 5C), including
548 two earlier reported alleles (Vandenbussche et al., 2003b), potentially yielding putative null
549 mutants for all four genes based on the insertion position of the *dTph1* transposon, either
550 disrupting the first exon encoding the MADS DNA binding domain or the K-region required
551 for protein-protein interactions in the case of the *euap1* allele. We obtained and analyzed
552 homozygous mutants for all insertion alleles, but all these homozygous mutants developed
553 normally (Figure 5C). Moreover, when we analyzed some double mutants to overcome putative

554 genetic redundancy, flowers in these double mutants developed normally, and inflorescence
555 architecture was not affected (Supplemental Figure 2B).

556

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

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

569 Quadruple *pfg fbp26 fbp29 euap1* mutants did display a severe phenotype in
570 inflorescence development. In fact, the normal cyme inflorescence architecture was completely
571 abolished, and instead a large number of leaves were produced from the main apical meristem
572 before terminating into a solitary flower (Figures 6E and 6G). In addition, branches that
573 developed from the base of the plant followed exactly the same developmental pattern (Figure
574 6L). The leaves produced on the main stem and side branches were generated in a spiral
575 phyllotaxy (Figures 6F and 6Q), characteristic of vegetative development (Figure 6P), in
576 contrast to the opposite positioning of bracts in a WT inflorescence meristem. Finally, after the
577 production of usually >25 leaves, this vegetative meristem was fully converted into a floral
578 meristem resulting in a solitary flower (Figures 6H to 6I) as opposed to the normal cyme
579 inflorescence structure in WT (Figure 6M). Note that quadruple mutant flowers consistently
580 displayed an increase in floral organ number (e.g. the flower shown in Figure 6A has 10 petals),
581 possibly because the full conversion of the vegetative meristem into a floral meristem resulted
582 in a larger floral meristem size. In addition, the corolla of these flowers was not always properly
583 organized, as the petal tube was often disrupted on one side.

584 Once this terminal flower was fully developed, new branches started to grow from
585 vegetative meristems that were present in the axils of the leaves further down on the stem
586 (Figure 6I). These branches produced again a large number of leaves before terminating with a
587 solitary flower (Figure 6X), after which the same process was repeated. Together these results

588 indicate that petunia *API/SQUA* genes are required to establish inflorescence meristem identity
589 and associated cymose branching of the petunia inflorescence.

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

595

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

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

622 role for petunia *API/SQUA* genes in repressing the B-function in the first whorl, together with
623 the *ROB/BEN* genes.

624

625 **DISCUSSION**

626 **A Comparison of *SEP/AGL6* and *API/SQUA* Functions in Petunia, Arabidopsis, Tomato** 627 **and Rice**

628 In this study, we exploited the natural *dTph1* transposon mutagenesis system in
629 petunia to identify mutants for all 11 members of the petunia *API/SEP/AGL6* superclade, and
630 created a series of higher order mutants to uncover putative redundant functions. Here we
631 discuss and compare our findings with the available functional data from mainly Arabidopsis,
632 tomato and rice (see Figures 1F and 5A for the composition of their *SEP/AGL6* and *API/SQUA*
633 subfamilies). Petunia and tomato on the one hand, and Arabidopsis on the other hand are
634 representatives of the Asterids and Rosids respectively, which constitute the two major groups
635 in the core eudicots, and are thought to have diverged >100 million years ago (Moore et al.,
636 2010). Comparison of the molecular mechanisms controlling flower development in these
637 species therefore helps to assess conservation and divergence of the floral regulatory gene
638 network in the core eudicots (Vandenbussche et al., 2016). Petunia and tomato both belong to
639 the Solanaceous family, and the lineages leading to petunia and tomato are estimated to have
640 diverged around 30 MYA (Bombarely et al., 2016). Their close relationship is indeed reflected
641 in a high degree of sequence similarity between tomato/petunia orthologous pairs in the
642 *API/SEP/AGL6* superclade (see also Supplemental Data Files 1, 2, 3 and 4), which makes the
643 petunia/tomato comparison particularly well suited to evaluate functional diversification
644 patterns on a shorter evolutionary time-scale, as opposed to the comparison with the distant
645 monocot model species rice.

646

647 **Implication of *SEP* and *AGL6* Gene Functions in Floral Organ Identity**

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

656 rescue WT development in a triple mutant background (Ditta et al., 2004). Thus *SEP3* seems
657 to perform a master SEP floral organ identity function in both species.

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

668 *Arabidopsis sep1 sep2 sep3* mutants display a full conversion of petals, stamens and
669 carpels into sepals, and flowers are fully indeterminate (Pelaz et al., 2000). By contrast, the
670 genetically equivalent *fbp2 fbp5 pmads12* mutant in petunia retains -albeit reduced- petal and
671 stamen tissues, and the basic organization of the placenta structure in the flower center is
672 maintained. Thus unlike in *Arabidopsis*, genes outside the *SEP3* and *SEP1/SEP2* clades are
673 able to rescue part of the B- and C-functions in a petunia *sep1/sep2/sep3* mutant background.
674 We identified petunia *AGL6* as one of these genes (Rijkema et al., 2009). Similarly, the two
675 rice *AGL6* genes *OsMADS6/MFO1* and *OsMADS17* perform *SEP-like* functions, partly in a
676 redundant fashion with the *SEP* gene *OsMADS1/LHS1* (Ohmori et al., 2009; Dreni and Zhang,
677 2016). More recently, a floral organ identity function was also proposed for the tomato *AGL6*
678 gene, based on RNAi (Yu et al., 2017). Despite that the *Arabidopsis* genome encodes two *AGL6*
679 homologs (*AGL6* and *AGL13*), the phenotype of the *sep1 sep2 sep3* mutant demonstrates that
680 *Arabidopsis AGL6* genes may have lost most of their SEP-like activity compared to petunia,
681 rice and tomato *AGL6* genes, in agreement with the diverse proposed functions for *Arabidopsis*
682 *AGL6* and *AGL13* (Koo et al., 2010; Huang et al., 2012; Hsu et al., 2014).

683
684 Furthermore, we showed that in a petunia sextuple *sep/agl6* mutant a full *sepallata*
685 phenotype was obtained, including complete loss of floral meristem termination. Remarkably,
686 the obtained phenotype was similar to that of the earlier described *FBP2* co-suppression line
687 (Ferrario et al., 2003), demonstrating the efficiency of co-suppression to silence multiple genes
688 simultaneously. The expression levels of all six petunia *SEP* genes (but not of *AGL6*) were
689 monitored in the co-suppression line, but only *FBP2* and *FBP5* were found to be

690 downregulated. This strongly suggests that other genes were silenced at the post-transcriptional
691 level as was reported to frequently occur in gene silencing experiments (Stam et al., 1997).
692 Measuring mRNA levels of paralogous genes thus appears to be a limited method to assess the
693 specificity of a silencing construct.

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

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

715 In petunia sextuple *sep/agl6* mutant flowers, we found that the initial expression levels
716 of the B-class genes *PhGLO1*, *PhGLO2* and *PhDEF* and of the C-class genes *PMADS3* and
717 *FBP6* were comparable to WT, indicating that initial activation and expression of these genes
718 does not depend on the *SEP/AGL6* floral organ identity function. In Arabidopsis, a similar
719 observation has been made, showing normal patterning and accumulation of *AP3*, *PI* and *AG*
720 expression in young *sep1 sep2 sep3* floral buds (Pelaz et al., 2000). With perhaps the exception
721 of *FBP6* (*SHPI/2*), we found that further upregulation during later stages of development was
722 impaired, especially for the *PI* homologs *PhGLO1* and *PhGLO2*, while *PhDEF* (*AP3*) and
723 *PMADS3* (*AG*) still showed upregulation, but with a smaller incremental rate. These results are

724 in agreement with the idea that in Arabidopsis, complex formation of SEP proteins with B- and
725 C- class MADS-box proteins is required for their positive autoregulation (Gomez-Mena et al.,
726 2005; Kaufmann et al., 2009).

727 In sharp contrast with the other B-class genes, *PhTM6* expression levels in the sextuple
728 mutant were almost completely abolished during all stages tested, indicating a full dependence
729 on SEP/AGL6 activity for all stages of its expression. Earlier, we showed that regulation of
730 *PhTM6* expression is atypical for a B-class gene, since its expression largely depends on the
731 activity of the C-genes *PMADS3* and *FBP6* (Heijmans et al., 2012), resulting in a WT
732 expression pattern mainly in stamens and carpels from early developmental stages onwards,
733 and in all floral whorls when the C-genes are ectopically expressed (Vandenbussche et al.,
734 2004; Rijpkema et al., 2006). Together, this indicates that both *SEP* and C-class genes are
735 absolutely required for *PhTM6* expression, most likely as interaction partners in a MADS-box
736 protein complex (Ferrario et al., 2003). For *FBP11* (*STK*) expression, we found the same *SEP*
737 dependence, but since *FBP11* is expressed relatively late during flower development in the
738 developing placenta and ovules (Angenent et al., 1995), this may also be an indirect effect,
739 since these tissues are completely absent in the sextuple mutant. Thus, B- and C-class MADS-
740 box proteins may have an absolute requirement of SEP function to activate their downstream
741 developmental programs, but depend only partly on it for upregulation of their own expression.
742 This suggests differences in the molecular mechanisms involved in autoregulation versus
743 downstream target gene activation/repression.

744 Finally, we found that *UNS*, a petunia member of the *SOCI* family, was strongly
745 upregulated in the sextuple *sep/agl6* mutant from early stages onwards, suggesting that *SEP*
746 genes repress *UNS* during WT flower development. *SOCI* was identified as a direct target of
747 SEP3 in a genome wide study in Arabidopsis, with the expression of *SOCI* being already
748 reduced after only 8h of *SEP3* induction in seedlings (Kaufmann et al., 2009). Interestingly, it
749 was shown that constitutive *UNS* expression in petunia and Arabidopsis flowers lead to the
750 *unshaven* floral phenotype, which is characterized by ectopic trichome formation on floral
751 organs and conversion of petals into organs with leaf-like features (Ferrario et al., 2004). All
752 these observations are consistent with the finding of Ó'Maoiléidigh and colleagues, who
753 demonstrated that the floral homeotic organ identity gene *AG* not only functions by positively
754 conferring floral identity to organ primordia in the flower, but also by actively repressing
755 components of the leaf developmental program (OMaoileidigh et al., 2013).

756
757

758 **The *FBP9* Subclade Genes together with a *SEP4-like Gene* are Required to Confer Floral**
759 **Meristem Identity in petunia and tomato.**

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

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

787 While the strongest phenotype was obtained in the tomato triple mutants, analysis of
788 single and double mutants revealed also individual contributions to tomato development: *LIN*
789 limits internode length and the number of flowers that develop per inflorescence, *EJ2*
790 negatively regulates sepal size, while both *J2* and *EJ2* are involved in the control of branching

791 of the tomato inflorescence (Soyk et al., 2017). In addition, *J2* is required for the development
792 of the pedicel abscission zone (Liu et al., 2014; Roldan et al., 2017; Soyk et al., 2017).

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

802

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

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

819

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

823 Because Arabidopsis *ap1* mutants lack petals and have sepals displaying bract like
824 features (Irish and Sussex, 1990; Mandel et al., 1992) and *API* is negatively regulated by AG

825 in whorls three and four (Gustafson-Brown et al., 1994), *API* has been classified as an A-
826 function gene in the ABC model, required for the identity specification of sepals and petals. In
827 sharp contrast with the phenotype of Arabidopsis *ap1* mutants, we found that petal development
828 does not at all require *euAPI* activity in petunia. This may not come as a complete surprise
829 since it was shown before that also in Arabidopsis, *API* is not essential for petal development,
830 as evidenced by the nearly complete restoration of petal development in *ap1 ag* mutants
831 (Bowman et al., 1993) and in 35S: *SEP3 ap1* flowers (Castillejo et al., 2005), and a partial
832 restoration in *ap1 agl24* double mutants (Yu et al., 2004). In addition, single *euap1* mutants
833 that still develop petals have previously been described in other species such as e.g. the *squa*
834 mutant in snapdragon (Huijser et al., 1992), the *pim* mutant in pea (Berbel et al., 2001; Taylor
835 et al., 2002), *mtap1* in *Medicago* (Benlloch et al., 2006; Cheng et al., 2018), and *mc* in tomato
836 (Vrebalov et al., 2002; Nakano et al., 2012; Yuste-Lisbona et al., 2016).

837 Restricting the activity of the floral homeotic B- and C-functions to their proper domains
838 is crucial for the correct development of the flower structure, and it appears that the molecular
839 mechanisms underlying these cadastral functions are much more diverse compared to the floral
840 organ identity functions (reviewed in (Monniaux and Vandebussche, 2018)). Here we
841 identified the petunia *API/SQUA* genes as repressors of the B-function in the first whorl, as
842 evidenced by the partial conversion of sepals into petaloid tissue in *pfg fbp26 fbp29 euap1*
843 mutants, and the strong enhancement of this phenotype in combination with mutations in the
844 *ROB* genes, which were previously identified as B-function repressors in the first whorl (Morel
845 et al., 2017). Such a phenotype has so far never been reported in flowers of Arabidopsis *ap1*,
846 *cal* or *ful* mutants, or any combination of these mutations (Ferrandiz et al., 2000). Nevertheless,
847 it was proposed that *API* in combination with *AGL24* (*AGAMOUS LIKE 24*) and *SVP* (*SHORT*
848 *VEGETATIVE PHASE*) represses both the B- and C-function genes during early phases of floral
849 development (Gregis et al., 2006; Gregis et al., 2009), but it is not clear if other Arabidopsis
850 *API/SQUA* genes would be also involved in this process and whether this is specific to the first
851 floral whorl. Finally in rice, deregulation of B- and C-expression patterns was observed in
852 *osmads14 osmads15/+* and *osmads14/+ osmads15* flowers (Wu et al., 2017b), suggesting that
853 these rice *API/SQUA* transcription factors are also involved in patterning the homeotic B- and
854 C-functions.

855 In summary, the observation that the petunia *API/SQUA* genes repress the B-function
856 in the first floral whorl but do not seem to be required for 2nd whorl petal development
857 demonstrates that petunia *API/SQUA* genes cannot be easily classified as “A-function” genes
858 according to the original definition of the A-function in the ABC model. Earlier, we

859 encountered the same difficulties when trying to integrate the function of the petunia *AP2*-like
860 transcription factors *AP2* and *ROB1-3* into a simple ABC model (Morel et al., 2017). This led
861 us to propose a modified model for petunia floral organ identity in which the original A-function
862 is replaced by a combinatorial function describing the cadastral (boundary setting) mechanisms
863 that pattern the floral B- and C-functions (Morel et al., 2017). The above described cadastral
864 function of the petunia *API/SQUA* genes during flower development perfectly fits into this
865 alternative model, and is also compatible with the proposed modified (A)BC model (Causier et
866 al., 2009), in which a more broadly defined (A)-function provides the genetic context in which
867 the B- and C-functions are active and regulates both their spatial and temporal expression
868 domains. Our findings for both the *API/SQUA* and *AP2-like* gene functions in petunia entirely
869 explain the struggles to translate the Arabidopsis definition of the A-function to distant
870 flowering species (Litt, 2007).

871

872 **Petunia *API/SQUA* Family Members Function in a Largely Redundant Fashion and are** 873 **Required for Inflorescence Meristem Identity.**

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

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

891 One of the striking aspects of the phenotype of quadruple *pfg fbp26 fbp29 euap1* mutants
892 is that these plants develop fully functional flowers, suggesting that floral meristem identity

893 does not require AP1/SQUA activity in petunia. Our finding that this function is apparently
894 taken care off by a specific subset of *SEP* genes fully fits this hypothesis. However, we can
895 currently not fully exclude that some residual AP1/SQUA activity remains in the *pfg fbp26*
896 *fbp29 euap1* mutants, possibly explaining the formation of terminal flowers. Especially the *pfg-*
897 *12* insertion allele potentially could be a hypomorphic allele, since an alternative startcodon is
898 present in the first exon (AA nr 8, Supplemental Data File 2) shortly after the transposon
899 insertion site. This could in theory lead to the production of a protein with an N-terminal
900 truncation of the MADS-domain, perhaps displaying some residual functionality. Other alleles
901 will have to be identified in the future to fully proof the hypothesis that floral meristem identity
902 in petunia does not require AP1/SQUA activity.

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

910 Interestingly, it was earlier found that the tomato *mc* mutants also play a role in
911 inflorescence meristem development, since *mc* inflorescences revert to vegetative growth after
912 forming two to three flowers. In addition, these flowers developed enlarged sepals and have an
913 incomplete pedicel abscission zone (Vrebalov et al., 2002; Nakano et al., 2012; Yuste-Lisbona
914 et al., 2016). Moreover, the implication of MC in the development of the pedicel abscission
915 zone is proposed to occur via a higher order MADS-box complex including the SVP-like
916 protein JOINTLESS (J), and J2/SLMBP21 a SEP FBP9 clade member . Except for the pedicel
917 abscission zone which does not exist in petunia, the *mc* phenotypes are reminiscent of what we
918 observed in petunia quadruple *apl* mutants, suggesting a conserved role in inflorescence
919 meristem identity and first whorl development. Because *mc* single mutants have a clear
920 phenotype on their own, it also shows that *MC* exhibits less functional overlap with the other
921 *API* family members compared to petunia. However, as suggested by the relative mildness of
922 the inflorescence meristem defect in *mc* mutants compared to the petunia quadruple mutants,
923 this does not exclude possible partial redundancy with one or more of the other tomato *API*
924 family members, something that still remains to be tested. RNAi mediated downregulation of
925 tomato *FUL1* and *FUL2* suggested a role for these genes in fleshy fruit ripening (Bemer et al.,
926 2012; Shima et al., 2013; Wang et al., 2014), indicating that the implication of *FUL* genes in

927 fruit development is conserved between tomato and Arabidopsis, despite that these two species
928 have very different fruit types (fleshy versus dry). Petunia on the other hand develops a dry
929 fruit capsule, but the implication of *API/FUL* members in its development remains to be
930 investigated.

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

943

944 **Functional Diversification Patterns in the *API/SEP/AGL6* Superclade during Angiosperm** 945 **evolution.**

946 Above, we compared *API/SEP/AGL6* functions between different species, mainly
947 focusing on Arabidopsis, petunia, tomato and rice, revealing important differences in the
948 functions performed by their respective members. Perhaps the most striking observation is that
949 a subclass of *SEP* genes (all belonging to the *LOFSEP* group) in petunia, tomato and possibly
950 also rice are required to confer floral meristem identity, while in Arabidopsis the floral meristem
951 identity function is mainly associated with members of the *API/SQUA* subfamily. It thus seems
952 that during angiosperm evolution, members of different subfamilies within the *API/SEP/AGL6*
953 superclade have evolved specialized/subfunctionalized roles either in floral organ identity or
954 inflorescence and/or floral meristem determination, providing further genetic support for the
955 monophyletic origin of the *API/SEP/AGL6* superclade. Within MADS-box subfamilies, it is
956 not unusual that functions have been distributed differently between paralogs in different
957 species. One of the first well documented cases concerns the C-function MADS-box subfamily,
958 showing that the canonical C-function is encoded by nonorthologous genes in Arabidopsis and
959 *Antirrhinum* (Causier et al., 2005). However, careful comparison of gene functions in the
960 *API/SEP/AGL6* superclade suggest that this random distribution of functions after gene

961 duplication has occurred also during the earlier phases of the evolution of the MADS-box gene
962 family, resulting in functions that are differently distributed beyond the subfamily level. In
963 addition, comparison between tomato and petunia indicates major functional differences that
964 have arisen on a relatively short evolutionary time-scale. Of note is the involvement of several
965 tomato *API/SEP/AGL6* members in the development of the pedicel abscission zone and in
966 compound leaf development, all processes that do not occur in petunia.

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

976

977 **MATERIALS AND METHODS**

978 **Plant Material, Genotyping and Phenotyping**

979 *Petunia* plants were grown in soil (FAVORIT-argile 10) either in a greenhouse (16 h day/8 h
980 night: natural light supplemented with Philips Sodium HPS 400W SON-T AGRO light bulbs;
981 55.000 lumens) or outside protected by an agricultural tunnel (from April to October), both
982 under conditions that depend on local seasonal changes (45.72°N 4.82°E), or in growth
983 chambers (settings: 16 h day 22°C /8 h night 18°C, 75W Valoya NS12 LED bars, light intensity
984 130 μE). The identification of the following *dTph1* transposon insertion alleles (Figures 1G and
985 5D) was described previously (current allele naming based on exact insert position; old allele
986 names in between brackets): *fbp2-332 (fbp2-1)*; *fbp2-440 (fbp2-2)*; *fbp4-44 (fbp4-2)*; *fbp4-55*
987 *(fbp4-3)*; *fbp5-129 (fbp5-1)*; *fbp9-110 (fbp9-1)*; *pfg-12 (pfg-1)*; *fbp26-76 (fbp26-1)*
988 (Vandenbussche et al., 2003b), and *agl6-118 (agl6-1)* (Rijkema et al., 2009). Note that the
989 previously determined insert positions for some of these alleles differ by a few nucleotides
990 compared to the data presented here, due to the imperfect manual sequencing method used at
991 that time combined with the characterization of only the right border of the transposon flanking
992 sequences, not taking the *dTph1* 8bp target site duplication into account. Also, it was mentioned
993 that homozygous *fbp9-1 (fbp9-110)* mutants exhibited aberrations in plant architecture during
994 the reproductive phase (Vandenbussche et al., 2003b). However, later outcrossing analysis of

995 the *fbp9-1* allele showed that this defect was closely linked to *fbp9-1*, but not due to the *fbp9-1*
996 insertion, as confirmed by the absence of this phenotype in the new *fbp9-7* and *fbp9-90* alleles.
997 The following alleles *fbp2-209*; *fbp4-23*; *fbp5-51*; *pm12-37*; *pm12-118*; *pm12-325*; *euap1-317*;
998 *fbp29-31*; *fbp29-123* and *fbp29-153* were identified by BLAST-searching our sequence-
999 indexed *dTph1* transposon flanking sequence database (Vandenbussche et al., 2008) that was
1000 enlarged with the addition of extra populations. Exact insert positions were determined by
1001 aligning the transposon flanking sequences with the corresponding genomic and coding
1002 sequences. The insertion alleles were named after their exact insert position, expressed in bp
1003 downstream of the ATG in the coding sequence (Figures 1G and 5D). Offspring of candidate
1004 insertion lines were grown and genotyped by PCR using gene specific primer pairs flanking the
1005 insertion site (Supplemental Table 2). The following thermal profile was used for segregation
1006 analysis PCR: 11 cycles (94°C for 15s, 71°C for 20s minus 1°C/cycle, 72°C for 30s), followed
1007 by 40 cycles (94°C for 15s, 60°C for 20s, 72°C for 30s). For all alleles, homozygous mutants
1008 were obtained in offspring of the originally heterozygous insertion mutants, either containing
1009 the original transposon insertion allele, or a stably inherited out-of-frame derived footprint
1010 allele that was confirmed by sequencing, fully maintaining the mutation. Insertion alleles that
1011 were used in crosses for higher order mutant analysis are indicated in red in Figures 1G and
1012 5D. The different insertion alleles were further systematically genotyped in subsequent crosses
1013 and segregation analyses. To test genetic interactions with the *rob* mutations (Figure 6), a *pfg*
1014 *fbp26 fbp29 euap1* mutant was crossed with the earlier described *rob1 rob2 rob3* mutant (Morel
1015 et al., 2017). Phenotypic analysis of all single and higher order mutants was focused and limited
1016 to the screening for defects in floral organ development and inflorescence architecture.

1017

1018 **Phylogenetic Analysis**

1019 The phylogenetic analyses shown in Figures 1F and 5A were conducted using the advanced
1020 PhyML/oneClick workflow at ngphylogeny.fr (Lemoine et al., 2019). Full-length protein
1021 sequences of either SEP/AGL6 (Figure 1F) or AP1/SQUA (Figure 5A) subfamily members
1022 from petunia, tomato, arabidopsis and rice (Supplemental Table 1) were first aligned using
1023 MAFFT (Kato and Standley, 2013) applying the following options: Data type: Autodetection;
1024 MAFFT flavor: auto; Gap extend penalty: 0.123; Gap opening penalty: 1.53; Matrix selection:
1025 no matrix; Reorder output? true. Output format: FASTA (Supplemental Data files 1 and 2).
1026 Next, alignment curation was done using BMGE (Criscuolo and Gribaldo, 2010) with the
1027 following options: Sequence coding: AA; matrix: BLOSUM; Estimated matrix BLOSUM: 62;
1028 Sliding windows size: 3; Maximum entropy threshold: 0.5; Gap Rate cut-off: 0.5; Minimum

1029 block size: 3 and 5 for Figures 1F and 5A respectively. Using the resulting BMGE files,
1030 Maximum Likelihood trees were calculated using PhyML (Lemoine et al., 2018) with the
1031 following settings: Data type: amino acids; Evolutionary model: LG; Equilibrium frequencies:
1032 ML/Model. Proportion of invariant sites: estimated; Number of categories for the discrete
1033 gamma model: 4; Parameter of the gamma model: estimated; Tree topology search: Best of
1034 NNI and SPR. Optimize parameter: Tree topology, Branch length, Model parameter; Statistical
1035 test for branch support: Bootstrap; Number of bootstrap replicates: 1000. Seed value used to
1036 initiate the random number generator: 123456. The tree was rendered using Newick Display
1037 (Junier and Zdobnov, 2010). For the visual representation of the SEP/AGL6 analysis (Figure
1038 1F), mid-point rooting was applied on the node separating SEP and AGL6 subfamilies, while
1039 for the AP1/SQUA analysis (Figure 5A), mid-point rooting was applied on the node separating
1040 rice from eudicot AP1/SQUA proteins.

1041

1042 **Imaging and Microscopy**

1043 Electron microscopy images were obtained as previously described (Vandenbussche et al.,
1044 2009) or by using a HIROX SH-1500 benchtop environmental electron microscope equipped
1045 with a cooled stage. Macroscopic floral phenotypes were imaged by conventional digital
1046 photography using a glass plate as a support and black velvet tissue around 10 cm below the
1047 glass plate in order to generate a clean black background. When needed, backgrounds were
1048 further equalized by removing dust particles and light reflections with Photoshop. Images in
1049 Figures 6H, 6I and 6M were photographed using a Zeiss Imager M2 microscope equipped with
1050 an AxioCam MRc camera (Zeiss).

1051

1052 **RT-qPCR Expression Analysis.**

1053 Total RNA was extracted using the Spectrum Plant Total RNA kit (Sigma Aldrich) and treated
1054 with Turbo DNA-free DNase I (Ambion). RNA was reverse transcribed using RevertAid M-
1055 MuLV reverse transcriptase (Fermentas) according to the manufacturer's protocol. PCR
1056 reactions were performed in an optical 384-well plate in the QuantStudio™ 6 Flex Real-Time
1057 PCR System (Applied Biosystems), using FastStart Universal SYBR Green Master (Roche), in
1058 a final volume of 10µl, according to the manufacturer's instructions. Primers (Supplemental
1059 Table 2) were designed using the online Universal ProbeLibrary Assay Design Center (Roche).
1060 Data were analyzed using the QuantStudio™ 6 and 7 Flex Real-Time PCR System Software
1061 v1.0 (Applied Biosystems). *Petunia ACTIN*, *GAPDH*, and *RAN* were used as reference genes.
1062 PCR efficiency (*E*) was estimated from the data obtained from standard curve amplification

1063 using the equation $E=10^{-1/\text{slope}}$. Relative expression (R.E.) values on the y-axes are the average
1064 of nine data points resulting from the technical triplicates of three biological replicates \pm sd and
1065 normalized to the geometrical average of three $E^{-\Delta Ct}$, where $\Delta Ct = Ct_{GOI} - Ct_{ACTIN, GAPDH}$ and
1066 *RAN*.

1067 The floral bud series (marked floral buds 1–3 in Figures 1F, 4G, 5C and Supplemental Figure
1068 1) are successive developmental stages of complete floral buds harvested from the same
1069 inflorescences (Figure 1E). Young bracts were harvested from the node bearing stage 3 flowers,
1070 while inflorescence stem tissue was collected from the internode connecting node stage 4 and
1071 stage 5 bearing flowers. For each biological replicate, corresponding stages harvested from
1072 three inflorescences were pooled. Stage 3 corresponds to flower buds with a diameter of ~5 mm
1073 and from which individual floral organs can be easily dissected by hand. All analyses showing
1074 expression in separate floral organ types are from this stage. Biological replicates of the
1075 different floral organ types were composed of pooled stage 3 organs harvested from three
1076 different flowers each time. Floral buds marked “2” (diameter ~2.5 mm) and “1” (diameter ~1.5
1077 mm) are younger stages and were harvested from the next two nodes produced after bud stage
1078 3. In addition to 1.5-mm buds, stage 1 also includes the inflorescence meristem and very young
1079 developing floral primordia subtended by bracts, which are attached to the base of the pedicel
1080 of the 1.5-mm bud. For the sextuple mutant flower buds analyzed in Figure 4G, developmental
1081 stages in relation to wild-type development were deduced based on their position on the
1082 inflorescence. Vegetative apices (including very small leaf primordia) were harvested from 3-
1083 week-old seedlings by manually removing cotyledons, roots, and developed leaves. Young leaf
1084 primordia were isolated from the same 3-week-old seedlings. Each biological replicate of the
1085 vegetative apices and young leaf primordia consisted of pooled material harvested from each
1086 time 10 seedlings. The root samples were obtained by pooling 10-15 actively growing 2 cm
1087 root tips per biological replicate.

1088

1089 Accession Numbers

1090 Sequence data for the genes that were functionally analyzed in this article can be found in the
1091 GenBank/EMBL libraries under accession numbers *FBP2* (M91666.1); *FBP5* (AF335235.1);
1092 *PMADS12* (AY370527.1); *FBP9* (AF335236.1); *FBP23* (AF335241.1); *FBP4* (AF335234.1);
1093 *Ph-AGL6* (AB031035.1); *PFG* (AF176782.1); *FBP29* (AF335245.1); *FBP26* (AF176783.1);
1094 *Ph-euAPI* (MK598839) (see also Supplemental Table 1).

1095

1096 **Supplemental Data**

1097

1098 **Supplemental Figure 1.** RT-qPCR Expression Analysis of the Petunia *UNSHAVEN (UNS)*
1099 Gene in WT.

1100 **Supplemental Figure 2.** Further Characterization of the Petunia *API/SQUA* family.

1101

1102 **Supplemental Table 1.** Gene Names, Synonyms and Accession Codes/Gene Models for
1103 Sequences Shown in Figures 1 and 5, in Supplemental Figures 2, and in Supplemental Data
1104 Files 1, 2, 3, and 4.

1105 **Supplemental Table 2.** Oligo Sequences Used in this Study.

1106

1107 **Supplemental Data File 1: MAFFT Multiple** Alignment of SEP and AGL6 Protein
1108 Sequences from Petunia (Ph), Tomato (Sl), Arabidopsis (At) and Rice (Os) in fasta format.

1109 **Supplemental Data File 2: MAFFT Multiple** Alignment of API/SQUA Protein Sequences
1110 from Petunia (Ph), Tomato (Sl), Arabidopsis (At) and Rice (Os) in fasta format.

1111 **Supplemental Data File 3:** SEP/AGL6 Newick tree file.

1112 **Supplemental Data File 4:** API/SQUA Newick tree file.

1113

1114 **Acknowledgments**

1115 M.V. was supported by a CNRS ATIP-AVENIR award and the French National Research
1116 Agency program DODO (ANR-16CE20-0024-03). We thank A. Lacroix, J. Berger and P.
1117 Bolland for plant care assistance, and V. Bayle for electron microscopy technical support
1118 performed at the PLATIM platform, IFR BioScience Lyon (UMS3444/US8). We thank A.
1119 Rijpkema for help with preliminary studies.

1120

1121 **Author Contributions**

1122 M.V. and P.M. conceived and designed the experiments. P.M., P.C., V.B. S.C., F.R., S.R.B.,
1123 C.T., J.Z., and M.V. performed the experiments. P.M. and M.V. analyzed the data. M.V., M.M.
1124 and P.M. wrote the manuscript.

1125

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1495 **FIGURE LEGENDS**

1496

1497 **Figure 1. Characterization of the Petunia SEP/AGL6 MADS-box Genes.**

1498 **(A)** Section through a WT petunia W138 flower showing inner whorls. **(B)** Petunia seedpod ~4
 1499 weeks post-pollination surrounded by green sepals. **(C)** SEM (scanning electron microscopy)
 1500 images of sepal, bract and leaf adaxial and abaxial epidermal surfaces. Bars = 50 μ m. **(D)**
 1501 Longitudinal sections of developing petunia floral buds showing the placenta developing from
 1502 the center of the floral meristem s = sepal; p = petal; st = stamen; c = carpel; pl = placenta. Bars
 1503 = 200 μ m. **(E)** W138 floral bud developmental stages for RT-qPCR analysis shown in (G),
 1504 dissected from the top of an inflorescence (inset), of which the large floral bud at the right is
 1505 just prior to opening. Numbers indicate sampled stages. 1 to 3 correspond to floral bud
 1506 diameters of ~1.5, 2.5 and 5 mm respectively. Stage 1 also includes very early flower primordia,
 1507 bracts and the inflorescence meristem. Bar = 1 cm. **(F)** Maximum Likelihood phylogenetic
 1508 analysis of the SEP and AGL6 subfamily members of *Petunia hybrida* (*Ph*), *Solanum*
 1509 *lycopersicum* (*Sl*), *Arabidopsis thaliana* (*At*) and *Oryza sativa* (*Os*). Bootstrap values marked
 1510 in red (expressed in %, based on 1000 replicates) supporting tree branching are indicated near

1511 the branching points. The scale bar represents number of substitutions per site. Accession codes
1512 for the corresponding sequences are shown in Supplemental Table 1. Naming of subfamilies
1513 and subfamily clades is based on previously described phylogenies for the SEP subfamily
1514 (Malcomber and Kellogg, 2005; Zahn et al., 2005; Yu et al., 2016). (G) RT-qPCR expression
1515 analysis of the petunia *SEP/AGL6* genes. Relative expression (R.E.) levels are plotted as the
1516 mean value of three biological and three technical replicates \pm SE, normalized against three
1517 reference genes (see Material and Methods). Expression levels were measured in vegetative
1518 tissues (green bars; infl. stem = inflorescence stem); entire floral buds (orange bars) from 3
1519 developmental stages shown in (E) and dissected floral organs (red bars) obtained from flower
1520 buds corresponding to stage 3. (H) Schematic representations of the gene structures and
1521 insertion alleles of the petunia *SEP* and *AGL6* genes and floral phenotypes of the corresponding
1522 insertion mutants used in further crosses. Black boxes and lines represent exons and introns
1523 respectively. All gene models start at the start codon and end at the stop codon. Scale bars =
1524 500 bp. Red triangles indicate positions of *dTph1* transposon insertions. Alleles are named after
1525 the exact insert position of the *dTph1* element in number of base pairs downstream of the ATG
1526 in the coding sequence. The names of the insertion alleles that have been selected for the
1527 creation of double and higher order mutants are marked in red.

1528

1529 **Figure 2. The Petunia *fbp2 fbp5 pm12* Mutant, Genetic Equivalent of the Arabidopsis *sep1***
1530 ***sep2 sep3* Mutant, Still Displays B- and C-function Floral Characteristics.**

1531 (A) to (H) Top view of flowers from WT, single, double and triple mutants of petunia
1532 *SEP1/SEP2/SEP3* homologs. All images are at the same magnification. (I) to (L) Side view of
1533 WT and mutant flowers sectioned through the middle. All images are at the same magnification.
1534 (M) Close-up of dissected third whorl organs (stamens). (N) Close-up of dissected fourth whorl
1535 organs (carpels). (O) to (Q) SEM images of the outer ovary surface. Scale bars = 100 μ m.

1536

1537 **Figure 3. Petunia Floral Meristem Identity Depends on *FBP9/FBP23/FBP4* Activity.**

1538 (A) to (C) and (E) to (G) Top and side view of WT, *fbp9 fbp23* and *fbp9 fbp23 fbp4* plants 13
1539 weeks after sowing. (D) and (H) Schematic representation of inflorescence phenotypes. (I) to
1540 (L) SEM images of inflorescence apices in WT and *fbp4 fbp9 fbp23* mutants. Br: bracts; Se:
1541 sepals; F: flower; Fm: Flower meristem; Im: Inflorescence meristem. Scale bars = 100 μ m. (M)
1542 to (Q) Inflorescence architecture of lower order mutants compared to WT and *fbp9 fbp23 fbp4*
1543 mutants. (R) to (W) Flower phenotypes of *fbp4*, *fbp23* and *fbp9* mutations in combination with
1544 *fbp2*. All flowers are at the same magnification.

1545

1546 **Figure 4. Characterization of the Sextuple *fbp2 fbp4 fbp5 fbp9 pm12 agl6* Mutant**
1547 **Compared to WT.** Genotypes in each panel are indicated as follows: sext: sextuple *fbp2 fbp4*
1548 *fbp5 fbp9 pm12 agl6* mutant. WT: wild-type.

1549 (A) and (B) Top view of young (A) and mature flower (B). (C) Dissected floral organs of a
1550 flower similar to the stage as indicated by the asterisk in (F). W# indicate whorl numbers. (D)
1551 Longitudinal section through an older flower similar to the stage as indicated by the double
1552 asterisk in (F). (E) SEM images of the epidermis of the four different floral whorls (indicated
1553 by W#) in WT (left panels) and the sextuple mutant (right panels). (F) Inflorescences showing
1554 flowers at various stages of development and aging. The arrows indicate an example where
1555 three consecutive fully developed flowers arose from a single floral meristem. Scale bars: 0.25
1556 cm in (A); 0.5 cm in (B, D); 1 cm in (C, F); 50 μ m in (E). (G) RT-qPCR expression analysis of
1557 the petunia floral homeotic genes in WT versus sextuple *fbp2 fbp4 fbp5 fbp9 pm12 agl6*
1558 mutants. Petunia genes are indicated and names of corresponding Arabidopsis orthologs are
1559 shown in between brackets. *No *TM6* ortholog exists in the Arabidopsis genome. **Petunia
1560 *FBP6* is orthologous to *SHP1/SHP2*, but is functionally homologous to *AG*. Relative expression
1561 (R.E.) levels are plotted as the mean value of three biological and three technical replicates
1562 \pm SE, normalized against three reference genes (see Material and Methods). Expression levels
1563 were measured in entire floral buds from three developmental stages as shown in Figure 1E.

1564

1565 **Figure 5. Characterization of the Petunia *API/SQUA* MADS-box Subfamily.**

1566 (A) Maximum likelihood phylogenetic analysis of the *API/SQUA* subfamily members of
1567 *Petunia hybrida* (*Ph*), *Solanum lycopersicum* (*Sl*), *Arabidopsis thaliana* (*At*) and *Oryza sativa*
1568 (*Os*). Bootstrap values marked in red (expressed in %, based on 1000 replicates) supporting
1569 branching are indicated near the branch points. The scale bar represents number of
1570 substitutions/site. Accession codes for the corresponding sequences are shown in Supplemental
1571 Table 1. Naming of subfamilies and subfamily clades is based on previously described
1572 phylogenies for the *API/SQUA* subfamily (Litt and Irish, 2003; Yu et al., 2016; Maheepala et
1573 al., 2019). (B) RT-qPCR expression analysis of the petunia *API/SQUA* genes. Relative
1574 expression (R.E.) levels are plotted as the mean value of three biological and three technical
1575 replicates \pm SE, normalized against three reference genes (see Material and Methods). See
1576 legend of Figure 1G for sample description. (C) Schematic representations of the gene
1577 structures and insertion alleles of the petunia *API/SQUA* genes and corresponding floral

1578 phenotypes of insertion lines used for further crosses and analyses. Figure Legend as in Figure
1579 1H.

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1582 **Figure 6. Petunia API/SQUA Family Members are Required for Inflorescence Meristem**
1583 **Identity and Repress the B-function in the First Floral Whorl.**

1584 (A) to (D) Flower phenotype of *pfg fbp26 fbp29 euap1* mutants. Some sepals and petals have
1585 been removed in (B) to reveal inner organs. (D) Enlarged sepals showing petaloid sectors
1586 displaying petal conical epidermal cells (inset SEM image). (E) to (G) Inflorescence phenotype
1587 showing an “inflorescence” with spirally organized leaves (F) ending in a single terminal flower
1588 (G). (L) Side branches developing from the basis of the plant exhibit an identical inflorescence
1589 phenotype. (H), (I) and (M) Longitudinal sections of the apex of an inflorescence in vegetative
1590 state (G), and of an inflorescence with terminal flower (I), compared to the apex of a WT
1591 inflorescence (M). Red asterisks in (I) indicate vegetative lateral meristems. (J) to (K)
1592 Enhanced homeotic sepal-to-petal conversion compared to (C) and (D). (N) and (O) unmodified
1593 sepals in *rob1 rob2/+ rob3* mutants (N) compared to WT (O). (P) and (Q) SEM images of a
1594 WT vegetative meristem before the onset to flowering compared to the apex of a *pfg fbp26*
1595 *fbp29 euap1* inflorescence prior to terminal flower formation as in (H). (R) Schematic
1596 representation of a *pfg fbp26 fbp29 euap1* inflorescence (right) compared to an intermediate
1597 inflorescence phenotype as shown in (T) to (V). (S) to (X) Inflorescence phenotypes of WT,
1598 quadruple and various triple mutant combinations after prolonged flowering. White arrows
1599 indicate positions of previous terminal flowers. Scale bars: 1 cm in (A-G; J-L; N-O; S-X); 100
1600 μm in (P-Q; H, I, M); 50 μm in inset in (D).

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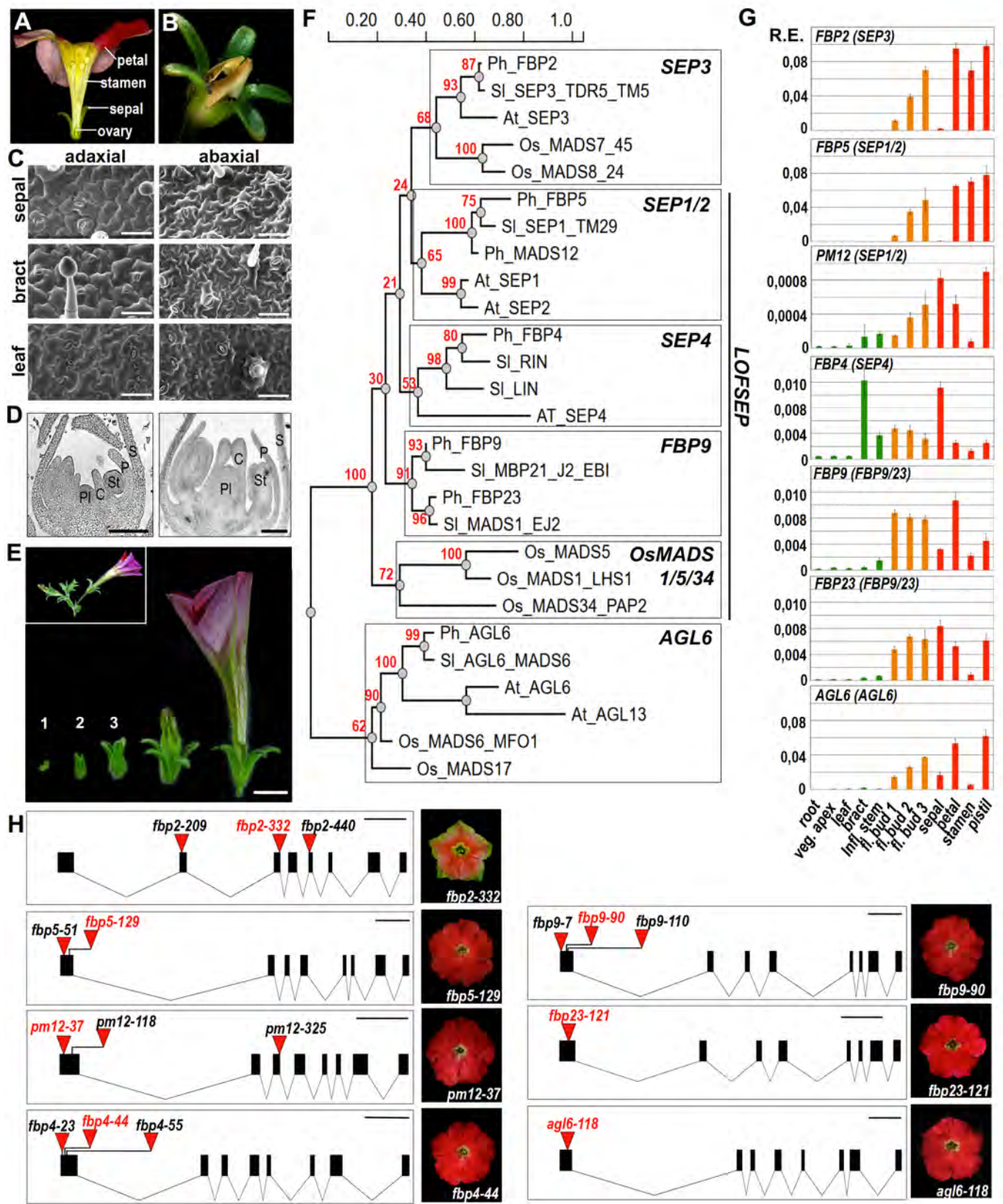


Figure 1. Characterization of the Petunia SEP/AGL6 MADS-box Genes.

(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 internal surfaces. Bars = 50 μ m. (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 μ m. (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 includes also 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* (Si), *Arabidopsis thaliana* (At) and *Oryza sativa* (Os). Bootstrap values marked in red (expressed in %, based on 1000 replicates) supporting tree branching are indicated near the branching points. The scalebar represents number of substitutions/site. Accession codes for the corresponding sequences are shown in Supplemental Table 1. Naming of subfamilies and subfamily clades are based on previously described phylogenies for the SEP subfamily (Malcomber and Kellogg, 2005; Zahn et al., 2005; Yu et al., 2016). (G) RT-qPCR expression analysis of the petunia SEP/AGL6 genes. Relative expression (R.E.) levels are plotted as the mean value of three biological and three technical replicates \pm SE, normalized against three reference genes (see Material and Methods). Expression levels were measured in vegetative 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 insertion alleles of the petunia SEP and AGL6 genes and floral phenotypes of the corresponding insertion mutants used in further crosses. Black boxes and lines represent exons and introns respectively. All gene models start at the start codon and end at the stop codon. Scale Bars = 500 bp. Red triangles indicate positions of *dTph1* transposon insertions. Alleles are named after the exact insert position of the *dTph1* element in number of basepairs downstream of the ATG in the coding sequence. The names of the insertion alleles that have been selected for the creation of double and higher order mutants are marked in red.

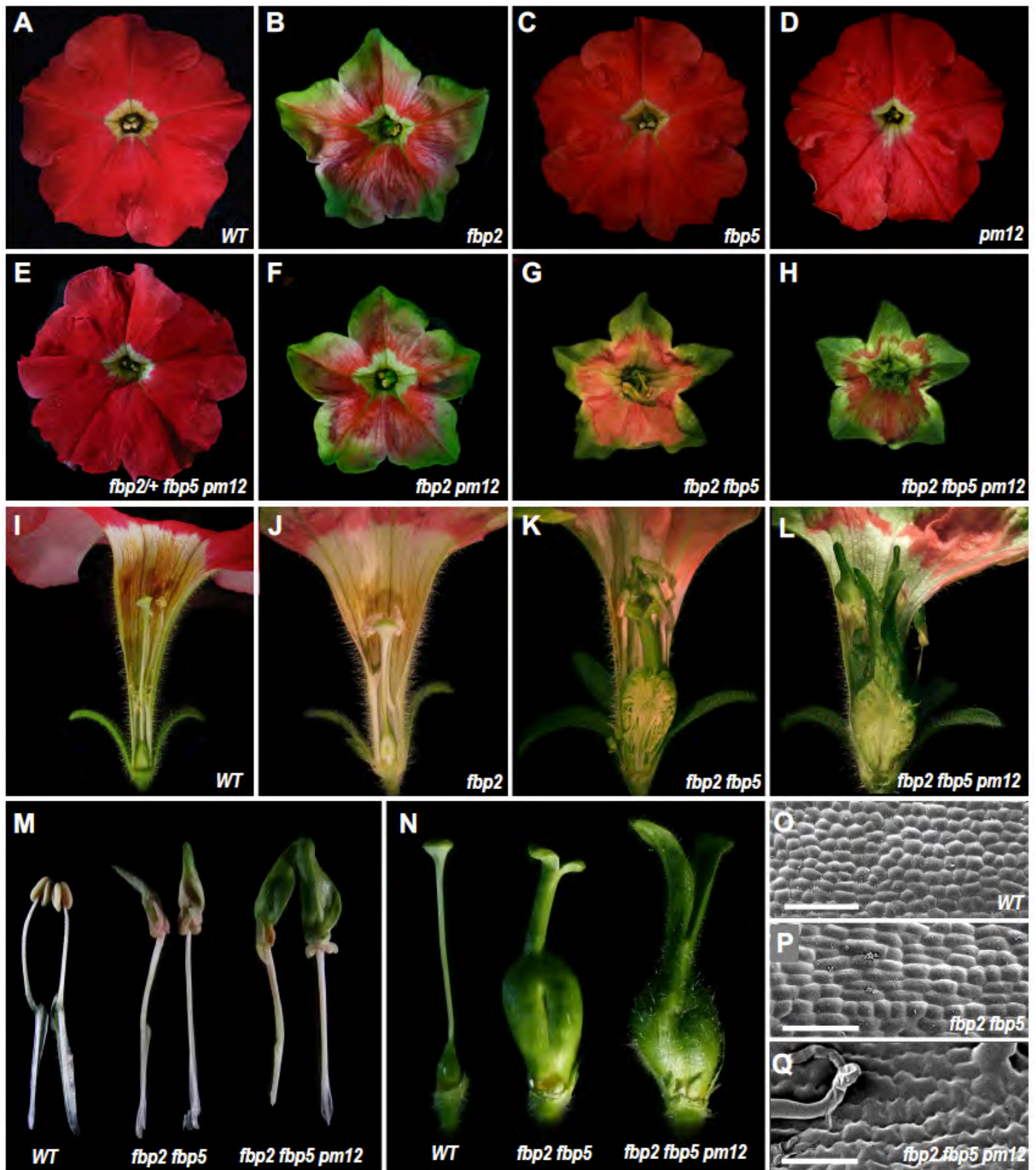


Figure 2. The Petunia *fbp2 fbp5 pm12* Mutant, Genetic Equivalent of the Arabidopsis *sep1 sep2 sep3* Mutant Still Displays B- and C-function Floral Characteristics. (A) to (H) Topview of flowers from WT, single, double and triple mutants of petunia *SEP1/2/3* homologs. All images are at the same magnification. (I) to (L) Sideview 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.

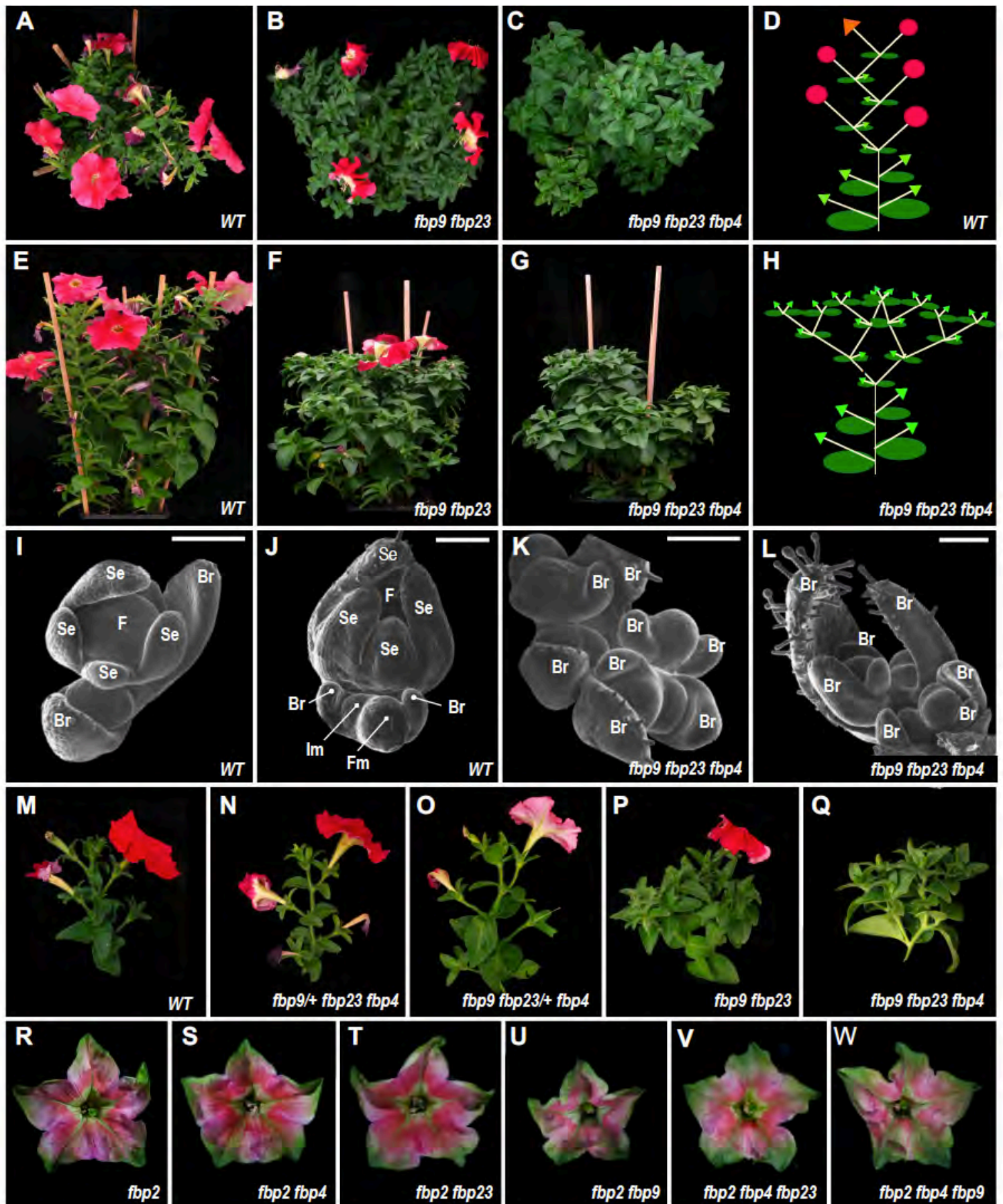


Figure 3. *Petunia* Floral Meristem Identity Depends on *FBP9/23/4* Activity.

(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 μ m. (M) to (Q) Inflorescence architecture of lower order mutants compared to WT and *fbp9 fbp23 fbp4* mutants. (R) to (W) Flower phenotypes of *fbp4*, *fbp23* and *fbp9* mutations in combination with *fbp2*. All flowers are at the same magnification.

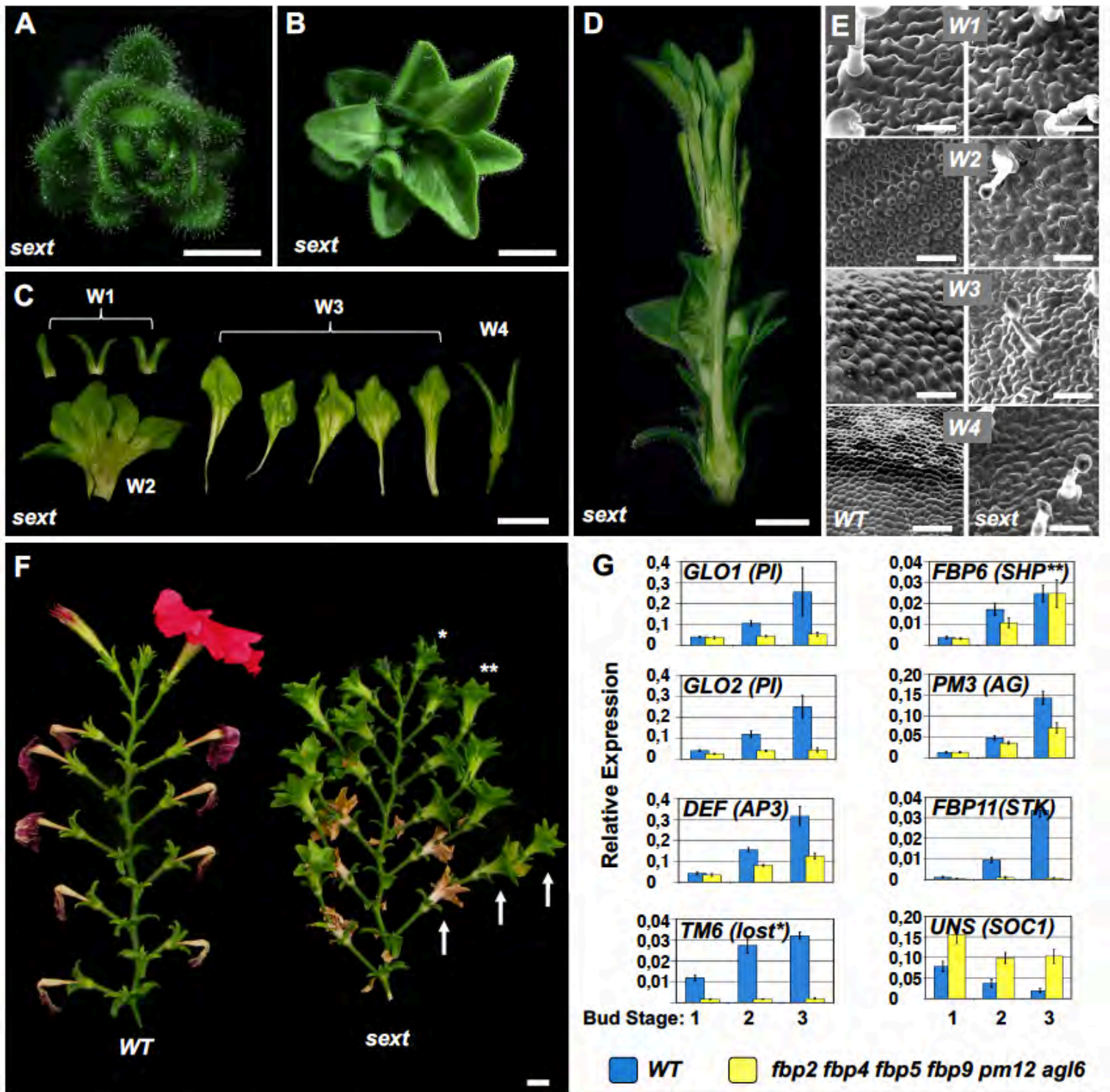


Figure 4. Characterization of the Sextuple *fbp2 fbp4 fbp5 fbp9 pm12 agl6* Mutant compared to WT. Genotypes in each panel are indicated as follows: *sext*: sextuple *fbp2 fbp4 fbp5 fbp9 pm12 agl6* mutant. WT: wild-type.

(A) and (B) Topview 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. Scalebars: 0,25 cm in (A); 0,5 cm in (B, D); 1cm in (C, F); 50 μm in (E). (G) RT-qPCR expression analysis of the petunia floral homeotic genes in WT versus sextuple *fbp2 fbp4 fbp5 fbp9 pm12 agl6* mutants. Petunia genes are each time indicated and names of corresponding Arabidopsis orthologs are shown in between brackets. *No *TM6* ortholog exists in the Arabidopsis genome. **Petunia *FBP6* is orthologous to *SHP1/2*, but is functionally homologous to *AG*. Relative expression (R.E.) levels were 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.

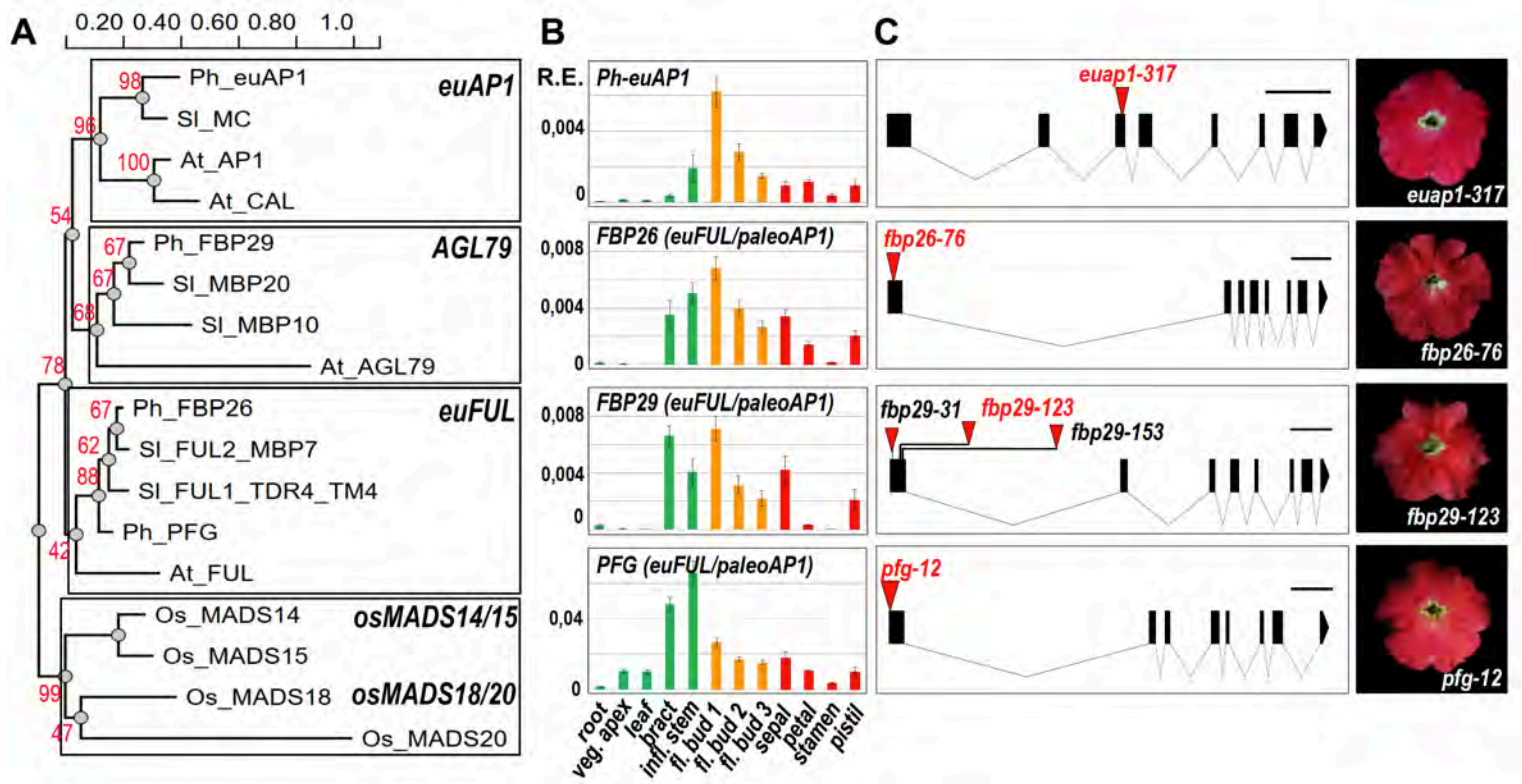


Figure 5. Characterization of the Petunia AP1/SQUA MADS-box Subfamily.

(A) Maximum Likelihood phylogenetic analysis of the AP1/SQUA subfamily members of *Petunia hybrida* (Ph), *Solanum lycopersicum* (SI), *Arabidopsis thaliana* (At) and *Oryza sativa* (Os). Bootstrap values marked in red (expressed in %, based on 1000 replicates) supporting tree branching are indicated near the branching points. The scalebar represents number of substitutions/site. Accession codes for the corresponding sequences are shown in Supplemental Table 1. Naming of subfamilies and subfamily clades are 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 AP1/SQUA genes. Relative expression (R.E.) levels are plotted as the mean value of three biological and three technical replicates \pm 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 AP1/SQUA genes and corresponding floral phenotypes of insertion lines used for further crosses and analyses. Figure Legend as in Figure 1H.

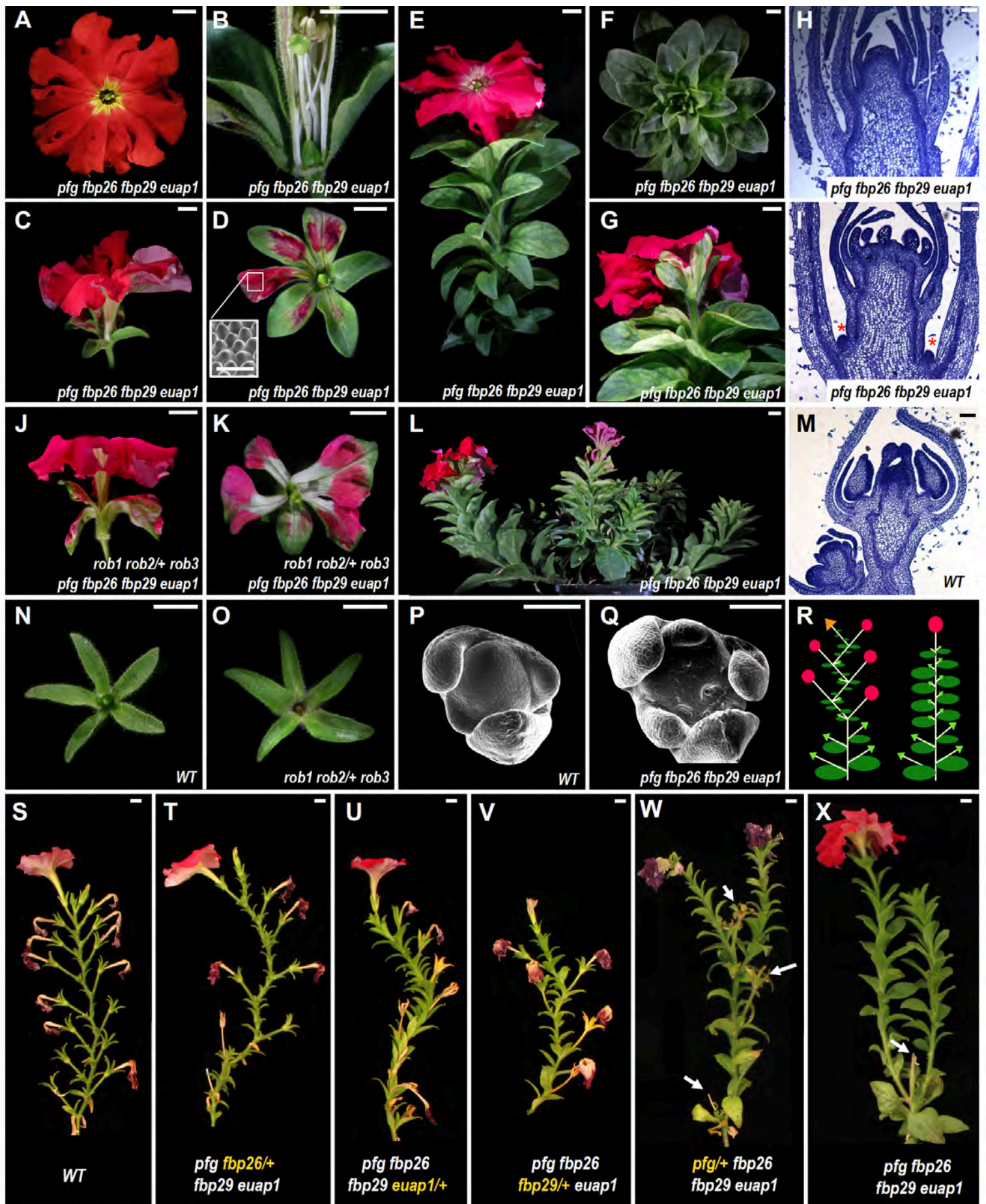


Figure 6. Petunia AP1/SQUA family members are Required for Inflorescence Meristem Identity, and Repress the B-function in the First Floral Whorl.

(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 μ m in (P-Q; H, I, M); 50 μ m in inset in (D).

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

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Plant Cell; originally published online October 7, 2019;
DOI 10.1105/tpc.19.00162

This information is current as of November 4, 2019

Supplemental Data	/content/suppl/2019/10/07/tpc.19.00162.DC1.html
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