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1 RESEARCH ARTICLE

2

3 **Cell layer-specific expression of the homeotic MADS-box transcription factor**
4 **PhDEF contributes to modular petal morphogenesis in petunia**

5

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16

17 **Short title:** Role of cell layers in petal development

18

19 **One-sentence summary:** The expression of B-class homeotic MADS-box transcription factor
20 *PhDEF* in different cell layers drives petunia petal tube or limb development.

21

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29

30 ABSTRACT

31 Floral homeotic MADS-box transcription factors ensure the correct morphogenesis of floral organs,
32 which are organized in different cell layers deriving from distinct meristematic layers. How cells
33 from these distinct layers acquire their respective identities and coordinate their growth to ensure
34 normal floral organ morphogenesis is unresolved. Here, we studied petunia (*Petunia x hybrida*)
35 petals that form a limb and tube through congenital fusion. We identified petunia mutants (periclinal
36 chimeras) expressing the B-class MADS-box gene *DEFICIENS* in the petal epidermis or in the
37 petal mesophyll, called *wico* and *star*, respectively. Strikingly, *wico* flowers form a strongly reduced
38 tube while their limbs are almost normal, while *star* flowers form a normal tube but greatly reduced
39 and unpigmented limbs, showing that petunia petal morphogenesis is highly modular. These
40 mutants highlight the layer-specific roles of PhDEF during petal development. We explored the link
41 between PhDEF and petal pigmentation, a well-characterized limb epidermal trait. The anthocyanin
42 biosynthesis pathway was strongly down-regulated in *star* petals, including its major regulator
43 *ANTHOCYANIN2* (*AN2*). We established that PhDEF directly binds to the *AN2* terminator *in vitro*
44 and *in vivo*, suggesting that PhDEF might regulate *AN2* expression and therefore petal epidermis
45 pigmentation. Altogether, we show that cell layerspecific homeotic activity in petunia petals
46 differently impacts tube and limb development, revealing the relative importance of the different
47 cell layers in the modular architecture of petunia petals.

48

49 IN A NUTSHELL

50 Background: Petals are not only beautiful, but they are also very important floral organs that have
51 co-evolved with different animal visitors to ensure pollination. This long co-evolution produced
52 many complex petal shapes. In the case of *Petunia*, the fused petals are organized in two domains,
53 the tube and the limb; this influences the interaction of the flower with hawkmoths, hummingbirds,
54 or bees. Petal identity genes, such as *PhDEFICIENS* (*PhDEF*), trigger petal development resulting
55 in mature petals. However, the mechanisms by which those genes drive complex petal shape with
56 tube and limb, are unclear.

57

58 Question: Petals are formed of cell layers: the epidermis and the internal cells. In a wild-type
59 flower, the petal identity gene *PhDEF* is expressed in all cell layers. But what happens if *PhDEF*
60 expression is restricted to a specific cell layer? In other words, we wanted to investigate the layer-
61 specific contribution of *PhDEF* in petal tube and limb development.

62 Findings: By chance, we obtained the perfect material to address this question: Two categories of
63 *Petunia hybrida* mutants (chimeras) expressing *PhDEF* exclusively in the petal epidermis or in the
64 inner cells, called *wico* and *star*, respectively. The resulting flowers displayed dramatically different
65 limb and tube shape (see picture): *wico* flowers form a strongly reduced tube while their limb is
66 almost normal, and *star* flowers form a normal tube but a very reduced limb. This suggests that

67 petunia petal morphogenesis is highly modular, and depends on the cell layer-specific expression of
68 *PhDEF*.

69 Next steps: This study is a first step towards understanding the link between *PhDEF* and complex
70 petal development. A major future challenge is to identify the genes acting downstream of the petal
71 identity genes, at the tissue (epidermis vs internal cells) and organ (limb vs tube) scales.

72

ACCEPTED MANUSCRIPT

73 INTRODUCTION

74 All plant aerial organs derive from clonally distinct layers, named L1, L2 and L3 in the shoot apical
75 meristem (SAM) (Satina et al., 1940). Within the L1 and L2 layers, cells divide anticlinally, thereby
76 maintaining a clear layered structure in all aerial organs produced by the SAM (Meyerowitz, 1997;
77 Stewart and Burk, 1970; Scheres, 2001). Already at the embryonic stage, meristematic cell layers
78 express different genes and have distinct identities (Abe et al., 1999; Lu et al., 1996), that are
79 maintained in the adult SAM (Yadav et al., 2014). During flower development, floral organ identity
80 will be appended on top of layer identity by the combinatorial expression of homeotic floral genes,
81 most of which are MADS-box genes (Coen and Meyerowitz, 1991; Schwarz-Sommer et al., 1990).
82 How these master floral regulators specify all floral organ features, such as organ size, shape,
83 pigmentation, and cellular properties, while maintaining layer-specific identities, is unknown.

84 Petals are often the most conspicuous organs of the flower, and they display a tremendous
85 diversity in size, shape and pigmentation across flowering plants (Moyroud and Glover, 2017).
86 Floral organ identity is specified by a combination of A-, B- and C-class identity genes as proposed
87 by the classical ABC model established in Arabidopsis (*Arabidopsis thaliana*) and snapdragon
88 (*Antirrhinum majus*), and B-class genes are particularly important for petal identity (Coen and
89 Meyerowitz, 1991; Schwarz-Sommer et al., 1990; Morel et al., 2017). B-class proteins, belonging to
90 MADS-box transcription factors, are grouped in the DEF/AP3 and the GLO/PI subfamilies, named
91 after the snapdragon/Arabidopsis B-class proteins DEFICIENS/APETALA3 and
92 GLOBOSA/PISTILLATA (Purugganan et al., 1995; Theißen et al., 1996). These proteins act as
93 obligate heterodimers consisting of one DEF/AP3 and one GLO/PI protein, together with other
94 MADS-box transcription factors of the SEPALLATA subfamily (Melzer et al., 2009), and this
95 complex activates the expression of *DEF/AP3* and *GLO/PI* genes for maintenance of high
96 expression levels throughout petal and stamen development (Tröbner et al., 1992).

97 In petunia (*Petunia x hybrida*, abbreviated *Ph* for gene names), gene duplication has
98 generated four B-class genes, namely *PhDEF* (*DEFICIENS*) and *PhTM6* (*TOMATO MADS-BOX*
99 *GENE6*) belonging to the *DEF/AP3* subfamily, and *PhGLO1* (*GLOBOSA1*) and *PhGLO2*
100 (*GLOBOSA2*) belonging to the *GLO/PI* subfamily (Vandenbussche et al., 2004; Rijpkema et al.,
101 2006; van der Krol et al., 1993; Angenent et al., 1992). Mutating the two members of each
102 subfamily (*phdef phtm6* or *phglo1 phglo2* double mutants) results in a classical B-function mutant
103 phenotype with homeotic transformation of petals into sepals and stamens into carpels
104 (Vandenbussche et al., 2004; Rijpkema et al., 2006). Additionally, gene copies within the *DEF/AP3*

105 subfamily have diverged in function: while *PhDEF* exhibits a classical B-class expression pattern
106 largely restricted to developing petals and stamens, *PhTM6* is atypically expressed in stamens and
107 carpels, and its upregulation depends on the petunia C-function genes (Rijkema et al., 2006;
108 Heijmans et al., 2012a). As a consequence, the single *phdef* mutant displays a homeotic conversion
109 of petals into sepals, while the stamens are normal due to functional redundancy with *PhTM6*
110 (Rijkema et al., 2006). The petunia *phdef* mutant is therefore an interesting model to study the
111 mechanism of petal identity specification alone since it displays a single-whorl complete homeotic
112 transformation, which is quite rare for floral homeotic mutants that generally show defects in two
113 adjacent whorls.

114 Flowers from the *Petunia* genus develop five petals, that arise as individual primordia and
115 fuse congenitally (Vandenbussche et al., 2009). Mature petals are fully fused and the corolla is
116 organized in two distinct domains: the tube and the limb. Variation in the relative size of the tube
117 and the limb is observed among wild species of *Petunia*, where flowers with a long tube grant
118 nectar access to long-tongued hawkmoths or hummingbirds, while wide and short tubes are easily
119 accessible to bees (Galliot et al., 2006). The short- and long-tube species cluster separately on a
120 phylogeny of wild *Petunia* species, and the short-tube phenotype is likely the ancestral one (Reck-
121 Kortmann et al., 2014). Pollinator preference assays and field observations have confirmed that tube
122 length and limb size are discriminated by pollinators and thereby might play a role in reproductive
123 isolation, together with multiple other traits of the pollination syndromes such as limb pigmentation
124 or volatile emission (Venail et al., 2010; Hoballah et al., 2007; Galliot et al., 2006). Tube and limb
125 therefore appear to act as different functional modules in the petunia flower.

126 Although the petunia petal tube and limb seem to play important ecological roles, the
127 mechanisms driving their development are mostly unknown. Tube and limb develop as relatively
128 independent entities in flowers from the Solanaceae family, to which petunia belongs: for instance,
129 tube length and limb width are uncorrelated traits in intra-specific crosses performed in *Nicotiana* or
130 *Jaltomata* (Bissell and Diggle, 2008; Kostyun et al., 2019). Moreover, tube and limb identities can
131 be acquired independently: this is strikingly observed in the petunia *blind* mutant, a partial A-class
132 mutant that forms an almost wild-type tube topped by functional anthers, due to ectopic C-class
133 activity in the second whorl (Cartolano et al., 2007). Apart from the petal identity genes, the
134 molecular players involved in petunia tube or limb morphogenesis are mostly unknown. General
135 growth factors affect petal development as a whole (both tube and limb) together with other
136 vegetative or reproductive traits (Vandenbussche et al., 2009; Terry et al., 2019; Brandoli et al.,

137 2020), but very few genes have been found to specifically affect growth of one subdomain of the
138 petal (Zenoni et al., 2004). Therefore, the mechanisms of petunia tube and limb morphogenesis
139 remain to be fully explored.

140 In contrast, the genetic and molecular bases of petunia petal pigmentation are extremely well
141 characterized, thanks to the plethora of mutants that have been isolated over decades of breeding
142 and research (Bombarely et al., 2016; Tornielli et al., 2009). Petunia limb pigmentation is mainly
143 due to the accumulation of anthocyanins in the vacuole of adaxial epidermal cells. Briefly, the
144 earliest steps of anthocyanin production are ensured by a MBW regulatory complex composed of an
145 R2R3-MYB transcription factor (either ANTHOCYANIN2 (AN2), AN4, DEEP PURPLE (DPL) or
146 PURPLE HAZE), a bHLH transcription factor (AN1 or JAF13), and a WD-40 repeat protein
147 (AN11), which drives the expression of anthocyanin biosynthesis enzymes and proteins involved in
148 vacuolar acidification of epidermal cells (Albert et al., 2011; de Vetten et al., 1997; Spelt et al.,
149 2000; Quattrocchio et al., 1998, 1999, 1993). How this pathway is activated, after regulators such as
150 PhDEF have specified petal identity, has not been elucidated so far.

151 In this work, we present petunia flowers with strongly affected tube or limb development,
152 that we respectively named *wico* and *star*, and that spontaneously arose from *phdef-151* mutant
153 plants. We provide genetic and molecular evidence that both of these flower types are periclinal
154 chimeras, resulting from the layer-specific excision of the transposon inserted into the *PhDEF* gene,
155 restoring *PhDEF* activity either in the epidermis or in the mesophyll of the petal. The *star* and *wico*
156 phenotypes indicate that in the petunia petal, the epidermis mainly drives limb morphogenesis while
157 the mesophyll mainly drives tube morphogenesis. This is seemingly different from previous studies
158 in snapdragon flowers, another species with fused petals, where *def* periclinal chimeras indicated
159 that epidermal *DEF* expression was making a major contribution to overall petal morphology
160 (Perbal et al., 1996; Vincent et al., 2003; Efremova et al., 2001). We characterized in detail the *star*
161 and *wico* petal phenotypes at the tissue and cellular scale, and found evidence for non-cell-
162 autonomous effects affecting cell identity between layers. We sequenced the total petal
163 transcriptome from wild-type (wt), *wico* and *star* flowers at three developmental stages, and we
164 found that a large proportion of the genes involved in anthocyanin production were downregulated
165 in *star* petal samples, as could be expected from their white petals. We further showed, by gel shift
166 assay and chromatin immunoprecipitation, that PhDEF binds to the terminator region of *AN2*,
167 thereby possibly regulating its expression and triggering the first steps of limb pigmentation. Our
168 results and our unique flower material promise to improve our understanding of tube and limb

169 morphogenesis in petunia, and address the broader question of how organ identity and cell layer
170 identity overlap during organ development.
171

ACCEPTED MANUSCRIPT

172 RESULTS

173 Spontaneous appearance of two phenotypically distinct classes of partial revertants from the 174 *phdef-151* locus

175 Previously described null alleles for the *PhDEF* gene (also named *GP* or *pMADSI*) were obtained
176 by either ethyl methanesulfonate (EMS) mutagenesis (de Vlaming et al., 1984; Rijpkema et al.,
177 2006) or by γ -radiation (van der Krol et al., 1993). Because neither of these alleles were
178 straightforward to genotype in a heterozygous state, we screened our sequence-indexed *dTph1*
179 transposon mutant population in the W138 genetic background (Vandenbussche et al., 2008) for
180 other insertions into *PhDEF*. We identified a mutant allele named *phdef-151*, referring to the *dTph1*
181 insertion 151 bp downstream of the ATG in the first exon of the *PhDEF* gene, predicted to fully
182 disrupt the MADS-domain in the protein sequence by premature termination of the first exon due to
183 multiple stop codons in the different reading frames of *dTph1*. As observed for previously identified
184 *phdef* null alleles, *phdef-151* flowers display a complete homeotic conversion of petals into sepals,
185 while heterozygous or homozygous wild-type siblings display red-coloured wild-type petals (Fig.
186 1A-C). *phdef-151* is thus very likely a null mutant allele.

187 While growing homozygous *phdef-151* individuals during several seasons, we repeatedly
188 observed the spontaneous appearance of inflorescence side branches that developed flowers with a
189 partial restoration of petal development (Figure 1, Supplemental Figure S1), suggesting excision of
190 the *dTph1* transposon from the *phdef-151* allele specifically in these side branches. Remarkably,
191 these partially revertant flowers could be classified as belonging to either one of two contrasting
192 phenotypic classes, that we named *star* and *wico*, and that could even occur simultaneously in
193 different branches on the same plant (Fig. 1A). For both phenotypic classes, we obtained more than
194 15 independent reversion events. The *star* flowers (Fig. 1D-F), named in reference to their star-
195 shaped petals, grow an elongated tube similar to wild-type (wt) flowers, but their limbs are
196 underdeveloped: they appear to mainly grow around the mid-vein with strongly reduced lateral
197 expansion, hence losing the typical round shape of wt limb. Moreover, they have almost white
198 petals, suggesting strongly reduced accumulation of anthocyanins.

199 We quantified the changes in flower morphology (Fig. 1K-N) and found that total limb area
200 was reduced almost 5-fold in *star* flowers (Fig. 1M). In contrast, total tube length was only slightly
201 reduced (by 19%) in *star* as compared to wt (Fig. 1L), and this was mainly due to a reduction in
202 length of domain D1, corresponding to the part of the tube fused with stamens (as defined in
203 (Stuurman et al., 2004), Fig. 1K), while length of the rest of the tube (domain D2) remained

204 unchanged (Fig. 1L, Supplemental Figure S2). As a result, the ratio between limb area and tube
205 length, which we use as a simple measure for overall corolla morphology, is reduced about 4-fold in
206 *star* flowers as compared to wt (Fig. 1N). In addition, we occasionally observed fully pigmented
207 secondary revertant sectors of various sizes in the *star* genetic background, in some cases leading to
208 the development of a single wt-like petal in a *star* flower background (Fig. 1J). These revertant
209 sectors, observed multiple times, always exhibited simultaneous restoration of pigmentation and
210 normal petal limb growth patterns, demonstrating that the strongly reduced pigmentation in *star*
211 petals was due to impaired PhDEF function, and not to an additional mutation in the pigmentation
212 pathway.

213 The *wico* flowers, named after their wide corolla, grow round-shaped and pigmented limbs
214 while their tube remains underdeveloped (Fig. 1G-I). Limb pigmentation ranged from pink to bright
215 red, and green sepaloid tissue was observed around the mid-veins, commonly well visible in all
216 *wico* flowers on the abaxial side of the petals (see for instance Supplemental Figure S1E). Total tube
217 length was reduced about 3-fold in *wico* flowers, with domain D1 being absent since stamens were
218 totally unfused to the tube (Supplemental Figure S2), while domain D2 was significantly reduced in
219 size compared to wild type (Fig. 1L). Limb area was also about 2-fold reduced in *wico* as compared
220 to wt flowers (Fig. 1M), but the ratio between limb area and tube length was higher than in wt
221 flowers (Fig. 1N), indicating the larger contribution of limb tissue to total corolla morphology in
222 *wico* flowers. In summary, the *star* flowers form an almost normal tube but small, misshaped and
223 unpigmented limbs, while the *wico* flowers form almost normally shaped and pigmented limbs but a
224 tube strongly reduced in length. These contrasting phenotypes suggest that tube and limb
225 development can be uncoupled in petunia flowers, at least to some degree.

226

227 **The *star* and *wico* flowers result from excision of the *dTph1* transposon from the *phdef-151***

228 **locus**

229 Reversion of a mutant phenotype towards a partial or a complete wt phenotype is classically
230 observed in unstable transposon insertion mutant alleles. In the petunia W138 line from which
231 *phdef-151* originates, the *dTph1* transposon is actively transposing (Gerats et al., 1990). We
232 assumed therefore that the *star* and *wico* flowers were caused by the excision of *dTph1* from the
233 *PhDEF* locus. *dTph1* transposition is generally accompanied by an 8-bp duplication of the target
234 site upon insertion, and excision can have various outcomes depending on the length and nature of
235 the remaining footprint (van Houwelingen et al., 1999). Hence, we first hypothesized that the

236 distinct *star* and *wico* phenotypes were caused by different types of alterations of the *PhDEF*
237 coding sequence after the excision of *dTph1*.

238 To test this hypothesis, we characterized the *phdef-151* locus from in total 14 *star* and 14
239 *wico* independent reversion events (Figure 2). For this, we amplified part of the *PhDEF* locus (Fig.
240 2A) and specifically sequenced the fragments resulting from *dTph1* excision in *phdef-151*, *star* and
241 *wico* second whorl organs (Fig. 2B-C). In *phdef-151*, the *dTph1*-excised alleles were always out-of-
242 frame, with either 7 or 8 additional nucleotides as compared to the wt sequence. Due to a reading
243 frame shift, both of these alleles are expected to produce an early truncated protein likely not
244 functional (Fig. 2C), in line with the normal *phdef* mutant phenotype observed in these plants. In
245 contrast, in both *star* and *wico* flowers we could find either wt sequences (found 1 time and 3 times
246 independently in *star* and *wico* flowers respectively) or in-frame footprint alleles consisting of
247 various additions of 6 nucleotides (alleles further named *PhDEF+6*, found 13 times and 11 times
248 independently in *star* and *wico* flowers respectively, Fig. 2C). These last insertions are predicted to
249 result in proteins with 2 additional amino-acids inserted towards the end of the DNA-binding
250 MADS domain (Fig. 2C). Together, these results demonstrate that *wico* and *star* revertant flowers
251 depend on the presence of an in-frame *def-151* derived excision allele that partially restores petal
252 development.

253 However, and in contrast to our initial expectations, there was no association between the
254 sequence of the locus after excision and the phenotype of the flower, and both *star* and *wico* flowers
255 could be found with a wt *PhDEF* excision allele or with an identical *PhDEF+6* allele (e.g. the 6-bp
256 GTCTGG footprint allele was frequently found both in *wico* and *star* flowers). This indicates that
257 the phenotypic difference between the *star* and *wico* flowers cannot be explained by a differently
258 modified *PhDEF* sequence after *dTph1* excision. Secondly, since the *phdef* mutation is fully
259 recessive (Vandenbussche et al., 2004), the presence of one transposon mutant allele combined with
260 the wt revertant sequence, normally should lead to wt flowers. Together this implied that another
261 molecular mechanism was causing the difference between *wico* and *star* flowers.

262

263 **The *wico* flowers are L1 periclinal chimeras**

264 Excision of *dTph1* from a gene can occur at different times during plant development: if happening
265 at the zygotic stage, then the whole plant will have a *dTph1*-excised allele. If excision occurs later,
266 this will result in a genetic mosaic (chimera) with a subset of cells carrying the *dTph1* insertion at
267 the homozygous state and others having a *dTph1*-excised allele. This typically leads to branches or

268 flowers with a wt phenotype on a mutant mother plant (assuming a recessive mutation).
269 Furthermore, since all plant organs are organized in clonally-independent cell layers, excision can
270 happen in one cell layer only, thereby creating a periclinal chimera, *i.e.* a branch or flower where
271 cell layers have different genotypes (Frank and Chitwood, 2016; De Keukeleire et al., 2001).

272 Analyzing the progeny of *wico* flowers suggested that they were periclinal chimeras, since
273 the *wico* phenotype was not heritable (in consequence, they had to be maintained by cuttings of
274 revertant branches). Instead, we found that the progeny of the *wico* flowers displayed a *phdef*
275 mutant phenotype at a proportion close to 100%, undistinguishable from the parental *phdef-151*
276 allele (Table 1). This suggested that the gametes generated by the *wico* flowers exclusively carried
277 the mutant *phdef-151* allele, hence resulting in homozygous *phdef-151* mutants in the progeny.
278 Gametes are exclusively derived from the L2 layer in flowering plants (Tilney-Bassett, 1986),
279 therefore indicating that L2-derived germ cells were homozygous mutant for *phdef-151* in *wico*
280 flowers, which should result in a *phdef* phenotype if the epidermal tissue had the same genotype.
281 This discrepancy suggested that the L1 layer of *wico* flowers was probably carrying a functional
282 *PhDEF* allele.

283 To test this hypothesis, we localized the *PhDEF* transcript in *wico* flowers by *in situ*
284 hybridization (Figure 3, Supplemental Figure S3). In wt flowers, the *PhDEF* transcript was first
285 detected in the stamen initiation domain, then shortly after in incipient stamen and petal primordia
286 (Fig. 3A, B). At all stages observed, *PhDEF* expression appeared quite homogeneous in all cell
287 layers of the organs, with a stronger expression in the distal part of the petal at later stages of
288 development (Fig. 3C, Supplemental Figure S3). In contrast, in *wico* flowers *PhDEF* expression
289 was restricted to the L1 and epidermis, all throughout petal development (Fig. 3G-I, Supplemental
290 Figure S3). Therefore, we conclude that *wico* flowers are the result of an early *dTph1* excision event
291 in one cell from the L1 meristematic layer, resulting in a chimeric flower expressing *PhDEF* only in
292 the epidermis (L1-derived cells) of petals. *Wico* flowers are therefore L1-periclinal chimeras.

293

294 **The *star* flowers are L2 periclinal chimeras**

295 Similarly, we analyzed the progeny of the *star* flowers, and the *star* phenotype was also not
296 heritable, and hence maintained by cuttings of revertant branches. The progeny of the *star* flowers
297 with a *PhDEF+6* allele yielded three different phenotypic classes (in a proportion close to 1:1:2;
298 Table 1): plants displaying a *phdef* phenotype, plants having wt flowers, and plants carrying flowers

299 with a wild-type architecture but with altered pigmentation, further referred to as « pink wt »
300 (Supplemental Figure S4).

301 We genotyped the *PhDEF* locus in plants descendant from one *star* parent and carrying
302 flowers with a wt architecture (Supplemental Table S1). We found that all plants with a pink wt
303 phenotype were heterozygous with an out-of-frame *phdef* allele and an in-frame *PhDEF+6* allele,
304 while fully red wt flowers had in-frame *PhDEF+6* alleles at the homozygous state. This indicates
305 that the PhDEF protein with 2 additional amino acids is not 100% fully functional, as it leads to a
306 reduction in limb pigmentation when combined with an out-of-frame allele. The fact that it can
307 ensure normal petal development when at the homozygous state indicates that this is dosage
308 dependent. In summary, the segregation ratio shows that the *star* gametes carried either the *phdef-*
309 *151* allele or an in-frame *PhDEF* allele at a 1:1 ratio, and hence that the germ cells generating these
310 gametes were heterozygous for these two alleles. This suggested that in *star* flowers, the L2 layer
311 was carrying a functional *PhDEF* allele (either wild-type *PhDEF* or *PhDEF+6*) while the L1 layer
312 was homozygous mutant for *phdef-151*.

313 In support of this, in *star* flowers *PhDEF* expression was absent from the L1 and epidermis
314 (Fig. 3D-F, Supplemental Figure S3). At the petal margins, underlying layers were also devoid of
315 *PhDEF* expression (Fig. 3F), which likely corresponds to the restricted petal area where cells of L1
316 origin divide periclinally and invade the mesophyll (Satina and Blakeslee, 1941). Therefore, we
317 conclude that *star* flowers are the result of an early *dTph1* excision event in one cell from the L2
318 meristematic layer, resulting in a chimeric flower expressing *PhDEF* only in the mesophyll (L2-
319 derived cells) of petals. *Star* flowers are therefore L2-periclinal chimeras. Considering the *star* and
320 *wico* phenotypes, we can conclude that the petal epidermis is the main driver for limb
321 morphogenesis (growth, shape and pigmentation), while the mesophyll mainly drives tube
322 morphogenesis (growth and shape).

323

324 **Non-cell-autonomous effects of layer-specific *PhDEF* expression on cell identity**

325 Having determined the genetic basis of the *star* and *wico* phenotypes, we next wondered how layer-
326 specific *PhDEF* expression affects the determination of cell identity, in the layer where *PhDEF* is
327 expressed (cell-autonomous effect) but also in the layer devoid of *PhDEF* expression (non-cell-
328 autonomous effect). For this, we observed petal adaxial epidermal cells by scanning electron
329 microscopy, and mesophyll cells on petal cross-sections, in wt petals and sepals, and in *star* and
330 *wico* petals (Figure 4).

331 On the adaxial side of the wt petal (Fig. 4A), cells from the limb are round and conical as in
332 many angiosperm petal limbs, while cells from the tube are elongated with a central cone (Fig. 4B)
333 (Cavallini-Speisser et al., 2021). In contrast, the adaxial epidermis of wt sepals (indistinguishable
334 from *phdef-151* second whorl organs) displays typical leaf-like features (Morel et al., 2019), with
335 puzzle-shaped cells interspersed with stomata and trichomes (Fig. 4B). Epidermal cell identity can
336 thus be clearly determined on the basis of cell shape. In *wico* petals, epidermal limb cells are
337 conical, similar to wt cells from the same area, although marginally bigger (Fig. 4B, D). In contrast,
338 cells from the tube, albeit displaying a similar shape to wt cells, are strongly reduced in length (Fig.
339 4B, E), suggesting that a defect in cell elongation is at least partly responsible for tube length
340 reduction in *wico* petals.

341 In *star* petal tubes, epidermal cells have a similar appearance as in a wt petal tube but are
342 slightly less elongated (Fig. 4B, E). In contrast, epidermal cells from the *star* limb are slightly
343 bulging cells, more or less roundish and about 3-times larger than wt conical cells (Fig. 4D).
344 Pigmented revertant sectors on *star* flowers (resulting from an additional *dTph1* excision in the
345 epidermis) allow the immediate comparison between *star* and wt epidermal cells on a single
346 sample, confirming the difference in conical cell size, shape and colour (Supplemental Figure S5).
347 Moreover, the *star* limb adaxial epidermis occasionally forms trichomes (Supplemental Figure S5),
348 a feature that is normally not observed in the wt limb adaxial epidermis. Altogether, these
349 observations suggest that epidermal cells from *star* limb have an intermediate identity between petal
350 and sepal cells.

351 Mesophyll cell identity was investigated by analyzing petal cross-sections stained with
352 toluidine blue (Fig. 4C). In the wt petal, mesophyll cells are loosely arranged, big and round in the
353 tube, and small and elongated in the limb. Sepal mesophyll cells are bigger than petal mesophyll
354 cells, and they display the typical leaf mesophyll organization with an upper palisade layer
355 (elongated and parallel cells) and a lower spongy layer (dispersed cells). Hence mesophyll cell size,
356 shape and tissue-level organization are characteristic features allowing to distinguish between sepal
357 and petal mesophyll tissue.

358 In *star* petals, the mesophyll strongly resembles a wt petal mesophyll in its organization,
359 however cells are bigger and more densely packed in the tube, suggesting that *PhDEF* activity in
360 the L2 layer is not entirely sufficient to specify normal mesophyll formation in the tube, which
361 might be linked to the slightly reduced size of the tube of *star* flowers (Fig. 1L). In *wico* petals,
362 mesophyll cells appeared very similar to wt and their organization was clearly distinct from the one

363 found in sepals since no palisade layer was observed. However, peeling the epidermis from *wico*
364 limb revealed that the underlying mesophyll harbored chloroplasts, similar to a sepal mesophyll and
365 in striking contrast with the white mesophyll of wt petal limb (Fig. 4F). Thus, the *phdef* mutant
366 mesophyll in *wico* flowers has an intermediate identity between sepal and petal. In summary, our
367 results show that for most features, *PhDEF* directs petal cell identity autonomously, and that non-
368 autonomous effects also influence cell identity across layers. The interpretation of these effects is
369 summarized in Supplemental Figure S6. In contrast, the observation of *star* revertant sectors
370 (Supplemental Figure S5) revealed that cell identity is entirely defined autonomously within the
371 epidermal layer, since a sharp transition in cell pigmentation, size and shape is observed in these
372 sectors (Supplemental Figure S5). This suggests that different processes are at stake for cell-cell
373 communication of petal identity across and within layers.

374 The physical nature of the non-autonomous effects that we identified remains unknown. Our
375 *in situ* hybridization assays show that the mRNA of *PhDEF* is not mobile, but our attempts to
376 localize the PhDEF protein by immuno-histochemistry have been unsuccessful; hence we do not
377 know if the PhDEF protein itself might move between petal layers. Alternatively, and non-
378 exclusively, other molecular players or mechanical signals might mediate information between
379 layers.

380

381 **Transcriptome sequencing of *star* and *wico* petals**

382 To better understand the molecular basis for the *star* and *wico* phenotypes, we performed RNA-Seq
383 on total petal tissue at three developmental stages, including wt and *phdef-151* samples (Figure 5).
384 We chose an early stage (stage 4 as defined in (Reale et al., 2002)), an intermediate stage (stage 8)
385 when tube length is at half its final size, and a late stage (stage 12) before limb is fully expanded
386 (Fig. 5A). For *phdef-151* we only sequenced second-whorl sepal tissue at stage 12 (before anthesis).
387 Principal component analysis showed that developmental stage is the first contributor to variation in
388 gene expression, while genotype corresponds to the second axis of variation (Fig. 5B). All
389 genotypes clustered separately except *wico* and wt samples which were highly similar at the two
390 later stages. We analyzed one-to-one differential gene expression between mutant and wt samples
391 with DESeq2 (Love et al., 2014) and we found on average 5,818 differentially expressed genes
392 (DEGs) in *phdef-151*, as compared to 1,854 and 1,115 DEGs in *star* and *wico* respectively, when
393 averaging for all stages (Fig. 5C, Supplemental Dataset S1).

394 There were generally more upregulated genes than downregulated ones in mutant or
395 chimeric genotypes, and the number of DEGs increased as development progressed in the petal in
396 both *star* and *wico* (Fig. 5C). At stage 12, a large proportion of DEGs (58-61%) in *wico* or *star*
397 petals were also differentially expressed in *phdef-151* (Fig. 5D), as expected since *wico* and *star*
398 flowers are mutant for *PhDEF* in one cell layer. Genes uniquely differentially expressed in *star* or
399 *wico* flowers represented 36% of DEGs for each, and only 16-29% of DEGs were jointly
400 differentially expressed in *star* and *wico* flowers, consistent with the very different phenotypes of
401 these flowers. These proportions indicate that the *star* and *wico* phenotypes are mostly subtended by
402 the differential expression of sets of genes also differentially expressed in *phdef-151*, together with
403 the differential expression of a unique set of genes for each genotype.

404 In *star* and *wico* petals, we found that *PhDEF* was down-regulated about two-fold at all
405 stages (Supplemental Figure S7), as expected since *PhDEF* is expressed in one cell layer only. In
406 contrast, *PhTM6* was not differentially expressed in *star* and *wico* nor in *phdef-151* (Supplemental
407 Figure S7), as expected since this atypical B-class gene is mostly expressed in stamens and carpels
408 and its upregulation depends on the C-function genes (Rijkema et al., 2006; Heijmans et al.,
409 2012b). Unexpectedly, we observed that the B-class genes *PhGLO1* and *PhGLO2* were not down-
410 regulated in *wico* petals, and only modestly in *star* petals, although their expression was almost null
411 in the *phdef-151* mutant (Supplemental Figure S7). The fact that *PhGLO1* and *PhGLO2* expression
412 does not strictly mirror the expression of *PhDEF* in *star* and *wico* petals, which is what we would
413 have expected since the B-class heterodimers are known to activate their own expression, suggests
414 that *PhGLO1* and *PhGLO2* expression is not entirely dependent on the B-class heterodimeric
415 complexes, in particular in the epidermal layer of the petal.

416

417 **PhDEF directly binds *in vivo* to the terminator region of AN2, encoding a major regulator of** 418 **petal pigmentation**

419 The *star* and *wico* periclinal chimeras have revealed layer-specific roles of PhDEF in the
420 establishment of petal identity and petal development. More specifically, the major layer-specific
421 phenotypes that we have identified are petal pigmentation, conical cell formation and limb growth
422 (controlled by the epidermal-specific expression of *PhDEF*), and tube growth (controlled by the
423 mesophyll-specific expression of *PhDEF*). Therefore, our chimeras show the potential to further
424 explore the exact nature of the link between layer-specific *PhDEF* activity and layer-specific
425 phenotypes. As a proof-of-concept, we explored if PhDEF could directly control petal pigmentation

426 in the limb epidermis. Pigmentation appeared to us as a trait of choice, since its regulatory and
427 biosynthetic factors are well described, while this was not the case for the other traits mentioned
428 above. Moreover, the absence of pigmentation in *star* petals, the restoration of pigmentation in L1-
429 revertant sectors and the phenotype of the pink wt flowers all converged to a direct link between
430 *PhDEF* expression in the epidermis and petal pigmentation.

431 For this, we examined the 451 genes down-regulated in both *phdef-151* and *star* samples (at
432 any stage) but not differentially expressed in *wico* samples (Supplemental Dataset S2), and we
433 found 23 anthocyanin-related genes in this gene set (Supplemental Figure S7), out of a total of 42 in
434 the whole genome, which constitutes an exceptionally high enrichment for this gene function ($p <$
435 0.001, Fisher's exact test). We paid particular attention to the genes possibly involved in the first
436 steps of anthocyanin production, ie encoding proteins involved in the MBW complexes activating
437 anthocyanin biosynthesis (*AN1*, *AN2*, *AN4*, *AN11*, *JAF13*, *DPL* and *PURPLE HAZE*). We found
438 that *ANI*, *AN2*, *DPL* and *JAF13* were downregulated both in *phdef-151* and *star* samples
439 (Supplemental Figure S7, Supplemental Dataset S2). *DPL* is involved in the limb venation pattern
440 (Albert et al., 2011; Zhang et al., 2021) and *JAF13* has only a moderate contribution to limb
441 pigmentation (Bombarely et al., 2016), therefore we decided to focus our attention on the two major
442 activators of anthocyanin biosynthesis *AN1* and *AN2* (Figure 6).

443 Indeed, the *an1* mutant has fully white petals and the *an2* mutant has strongly reduced limb
444 pigmentation (Quattrocchio et al., 1999; Spelt et al., 2000). Furthermore, *AN2* was shown to act as
445 an upstream activator of *ANI* since overexpressing *AN2* in petunia leaves is sufficient to activate
446 *ANI* expression, and for anthocyanins to accumulate (Quattrocchio et al., 1998; Spelt et al., 2000).
447 We observed that both genes were already expressed at stage 4 of wt petal development, before any
448 pigmentation is visible, and their expression levels strongly increased from stage 4 to stage 12,
449 while both being strongly downregulated in *star* petals and *phdef-151* second whorl organs, but not
450 in *wico* flowers (Fig. 6A, B). *AN2* was expressed at higher levels than *ANI* at all stages, consistent
451 with its most upstream role in the anthocyanin pigmentation pathway.

452 We aimed to test if PhDEF could directly bind to *ANI* and *AN2* genomic sequence,
453 potentially to regulate their expression. For this, we first attempted to predict PhDEF binding on the
454 genomic sequences of *ANI* and *AN2*. We used the high-quality transcription factor (TF) binding
455 profile database Jaspar (Fornes et al., 2020; Sandelin et al., 2004), using position weight matrices
456 for each TF to compute relative binding scores that reflect *in vitro* binding preferences (Stormo,
457 2013). The exact DNA-binding specificity of PhDEF has not been characterized, but that of its

458 Arabidopsis homologs AP3 and PI has been (Riechmann et al., 1996b). However, since PhDEF
459 DNA-binding specificity might be slightly different to those of AP3 and PI, we decided to predict
460 binding for all MADS-box TFs available in Jaspar 2020, accounting for 23 binding profiles
461 including those of AP3 and PI (Fornes et al., 2020). We hypothesized that sequences predicted to be
462 bound by several MADS-box TFs were putative CARG boxes (the binding site for MADS-box
463 proteins, whose canonical sequence is CC(A/T)₆GG, but real binding sites show some variation to
464 this consensus (Aerts et al., 2018)).

465 As a validation of this strategy, we analyzed the genomic sequence of *PhDEF* and found a
466 putative CARG box in the *PhDEF* promoter (visible by the presence of good predicted binding sites
467 for several MADS-box proteins and therefore appearing as a clear black line in Fig. 6C). This CARG
468 box has been validated in the literature: it is highly conserved between distantly-related flowering
469 plants (Rijpkema et al., 2006) and it was shown to be important for *AP3* petal-specific expression
470 and for its auto-activation in Arabidopsis (Hill et al., 1998; Wuest et al., 2012), and for DEF
471 function and binding to its own promoter in Antirrhinum (Schwarz-Sommer et al., 1992). We next
472 applied this predictive approach to the genomic sequences of *ANI* and *AN2*. For *ANI*, we predicted
473 a putative CARG box (*ANI-bs1*) with a very high score for several MADS-box proteins and for AP3
474 and PI in particular, in the terminator region (Fig. 6D). For *AN2*, we also predicted one putative
475 CARG box (*AN2-bs3*), again in the terminator region of the gene (Fig. 6E), although its binding
476 score was more modest in comparison to *ANI-bs1*. The sequence of *ANI-bs1* corresponds to a
477 close-to-canonical CARG box (CTATATTGG) and the sequence of *AN2-bs3* corresponds to a
478 perfectly symmetrical canonical CARG box (CCATAATAGG).

479 To determine if PhDEF could indeed bind to *ANI-bs1* and *AN2-bs3* and potentially regulate
480 *ANI* and *AN2* expression, we performed gel shift assays using *in vitro* translated PhDEF and/or
481 PhGLO1 proteins (Fig. 6F). We found that, when incubating a 60-bp fragment containing *ANI-bs1*
482 in its center with either PhDEF or PhGLO1, no shift in migration was visible, indicating that neither
483 protein could bind to this site alone. However, when incubating *ANI-bs1* with both PhDEF and
484 PhGLO1 proteins, we observed a clear shift in migration, consistent with the obligate
485 heterodimerization of these proteins necessary for DNA binding (Riechmann et al., 1996a).
486 Similarly, a 60-bp fragment containing *AN2-bs3* in its center, incubated with PhDEF and PhGLO1
487 proteins, resulted in a clear shift in migration. In contrast, a control 60-bp fragment named *ANI-bs2*,
488 located in the *ANI* terminator region but predicted to have a very low binding score (relative score
489 under 0.8 both for AP3 and PI), was not bound by the PhDEF + PhGLO1 protein complex, showing

490 that our assay was specific. Therefore PhDEF, when dimerized with PhGLO1, is able to bind to
491 sites in putative regulatory regions in *AN1* and *AN2*, suggesting that it might directly regulate the
492 expression of these two genes.

493 Next, we tested if PhDEF could bind *in vivo* to genomic regions containing *AN1-bs1* and
494 *AN2-bs3* by chromatin immunoprecipitation (ChIP). We produced recombinant PhDEF protein
495 devoid of its highly conserved MADS domain, to avoid cross-reactivity with other MADS-box
496 proteins, and generated a polyclonal antibody against this truncated PhDEF protein. We performed
497 the ChIP assay on second whorl organs (petal or sepal) from wt, *phdef-151* or *phglo1 phglo2* plants
498 at an intermediate stage of development (stage 8). In wt petal samples, we found a significant
499 binding enrichment for some of the genomic fragments (GF) that we tested, and in particular
500 *PhDEF^{GF1}* (Fig. 6G), containing the validated CARG box previously described (Fig. 6C), which is
501 expected since PhDEF activates its own expression.

502 We also observed a significant binding enrichment in *AN2^{GF3}* (Fig. 6G), containing the
503 previously identified *AN2-bs3* binding site (Fig. 6E). In contrast, no strong enrichment was detected
504 in the *AN1* genomic fragment containing the *AN1-bs1* strong *in vitro* binding site for PhDEF
505 (*AN1^{GF3}*). Our ChIP assay was specific, since no enrichment was detected for the *phdef-151* mutant,
506 nor for the *phglo1 phglo2* mutant (Fig.6G). The *phglo1 phglo2* samples constitute an indirect
507 control for PhDEF binding, since the PhDEF protein partners PhGLO1/PhGLO2 are absent, thereby
508 indirectly preventing PhDEF binding on DNA. The fact that we do not detect any binding
509 enrichment in these plants shows that our ChIP assay is robust. Therefore, we conclude that PhDEF
510 binds to the terminator region of *AN2* *in planta*, and that PhDEF is a putative direct regulator of
511 *AN2* expression in the petal epidermis.

512 **DISCUSSION**

513 In this work, we identified periclinal chimeras expressing the B-class MADS-box gene *PhDEF* in
514 different cell layers of the flower. This layer-specific expression resulted in the correct development
515 of sub-domains of the petal only, showing that epidermal *PhDEF* expression mainly drives limb
516 morphogenesis while its expression in the mesophyll is more important for tube morphogenesis.
517 This indicates that cell layer-specific actions of PhDEF are different and contribute in a
518 complementary fashion to overall petal development.

519

520 **Contribution of cell layers to mature petunia petals**

521 The SAM of all flowering plants is organized in three independent layers. Generally, it is assumed
522 that L1-derived cells form the epidermis, L2-derived cells produce the mesophyll and sub-
523 epidermal tissue, and L3-derived cells generate the ground tissues (inner mesophyll, vasculature,
524 pith of the stem). However, there is variation to this general pattern between organs; for instance
525 Arabidopsis sepals, stamens and carpels derive from these three layers, while petals derive from the
526 L1 and L2 layers only (Jenik and Irish, 2000). Moreover, the contribution of cell layers can vary
527 between the same organ in different species: for instance, petals from *Datura stramonium* (member
528 of the Solanaceae family like petunia) are derived from all three layers, in contrast to petals from
529 Arabidopsis (Satina and Blakeslee, 1941). Finally, even in one organ from a single species, cell
530 layer contribution is not always homogeneous in different parts of the organ: in *Datura* petals, the
531 L3 only participates in the vasculature at the base of the organ but does not contribute to the distal
532 part of the petal, and the L1 invades the mesophyll at the petal edges (Satina and Blakeslee, 1941).

533 In fact, the contribution of cell layers to mature organ organization can only be strictly
534 assessed by clonal analysis, where one follows cell lineage using trackable cell-autonomous
535 markers. In petunia, no clonal analysis has been performed so far, hence one can only assume which
536 cell layers participate in petal development based on clonal analyses performed in closely-related
537 species. In *Datura*, periclinal chimeras induced by colchicine treatment and refined histological
538 observations have provided a detailed clonal analysis for cell layers in floral organs (Satina and
539 Blakeslee, 1941). The first visible event of petal initiation is a periclinal cell division from the L2
540 layer, and further growth of the petal depends primarily on cell divisions from the L2, both
541 anticlinal and periclinal. The L3 layer only contributes to the vascular tissue at the very base of the
542 petal. L1-derived cells form the epidermis by anticlinal divisions, except at the petal edges where
543 periclinal divisions are observed, leading to L1-derived cells invading the mesophyll. Hence, the

544 *Datura* petal is formed by all 3 layers with a major contribution of the L1 and L2 layers, and a
545 relative enrichment in L1-derived cells (by thinning of the mesophyll) progressing from the base
546 towards the tip of the petal. In this work, we hypothesized that the petunia petal is formed similarly.
547 Accordingly, we only obtained two phenotypic classes of periclinal chimeras, *star* and *wico*,
548 suggesting that L3-specific *PhDEF* expression probably only leads to a *phdef* mutant phenotype.

549 The contribution of L1- and L2-derived tissues is heterogeneous in the petunia petal. Indeed,
550 cross-sections in the middle of the petal tube indicate that the mesophyll is thick, with several layers
551 of cells (Fig. 4C). The mesophyll tissue is quite dense in this part of the tube, with lacunae between
552 cells being relatively small. In contrast in the limb, mesophyll cells are very small and interspersed
553 with large lacunae. There is a general thinning of the mesophyll as we progress from the base of the
554 petal towards its edges, whereas the epidermis always appears as a single layer of tightly connected
555 cells. Therefore, the general contribution of cell lineages (L1- or L2-derived) to the petunia petal
556 explains to a large degree the *star* and *wico* phenotypes. Indeed, the limb is mostly derived from the
557 L1 layer, and therefore recovery of this lineage in the *wico* flowers is sufficient to restore limb
558 development. Similarly, the tube is composed of a much higher proportion of mesophyll than
559 epidermis cells, and recovery of the mesophyll lineage in the *star* flowers is sufficient to restore
560 tube development.

561

562 **Different cell layers drive tube and limb morphogenesis**

563 The *star* and *wico* phenotypes revealed that in petunia petals, the epidermis is the main driver for
564 limb morphogenesis while the mesophyll is the main driver for tube morphogenesis. The epidermis
565 has been proposed to be the layer in control of organ morphogenesis, since it is a layer under
566 tension that restricts growth of the underlying inner tissues that tend to expand (Kutschera and
567 Niklas, 2007). In particular, epidermal expression of the brassinosteroid receptor BRI1
568 (BRASSINOSTEROID INSENSITIVE 1) is sufficient to restore normal leaf morphogenesis in a
569 *bri1* mutant (Savaldi-Goldstein et al., 2007). Similarly, the expression of the auxin transporter PIN1
570 (PIN-FORMED 1) in the L1 of the SAM is sufficient to restore normal phyllotaxis in a *pin1* mutant
571 (Kierzkowski et al., 2013). However, pieces of evidence suggest that organ inner layers can have an
572 active role in morphogenesis: for instance, mesophyll-specific expression of *ANGUSTIFOLIA* (*AN*)
573 is sufficient to restore normal leaf width in the Arabidopsis *an* mutant (Bai et al., 2010); leaf shape
574 is controlled by the L2- and L3-derived tissues in *Nicotiana glauca* (McHale and Marcotrigiano,
575 1998); and the leaf mesophyll is the main player for leaf flatness in Arabidopsis (Zhao et al., 2020).

576 Moreover, expressing *BRI1* in the root phloem also restores *bri1* plant dwarfism (Graeff et al.,
577 2020). The contribution of cell layers to organ morphogenesis is thus a complex process that varies
578 between organs, species and the genetic systems investigated.

579 Our work has confirmed that the petunia petal has a modular structure, since tube and limb
580 can develop relatively independently from each other in the *star* and *wico* flowers. This modularity
581 is consistent with previous observations in the literature (described in the Introduction), and in line
582 with the different ecological roles of the tube and the limb for the interaction with pollinators. Our
583 results highlight that a homeotic factor, PhDEF, can participate in the establishment of this modular
584 structure. Indeed, although PhDEF is normally present in all cell layers of the wild-type petal, its
585 action in the different cell layers is mainly responsible for tube or limb development. This provides
586 a possible mechanism, at the tissue level, for the establishment of the modular structure of petunia
587 petals by homeotic genes. It also contributes to the understanding of how homeotic genes can
588 specify at the same time the overall identity of an organ and the coordinated development of its
589 different functional modules.

590 One may wonder if our findings apply outside of petunia flowers. In snapdragon and
591 Arabidopsis flowers, periclinal chimeras for orthologs of *PhDEF* (*DEF* and *AP3* respectively) or
592 *PhGLO1/PhGLO2* (*GLO* and *PI* respectively) have been previously obtained (Perbal et al., 1996;
593 Vincent et al., 2003; Efremova et al., 2001; Bouhidel and Irish, 1996; Jenik and Irish, 2001;
594 Urbanus et al., 2010b). In snapdragon, expression of *DEF* only in the L1 layer largely restores petal
595 development, particularly in the limb, in contrast to the L2/L3 specific *DEF* or *GLO* expression
596 which causes reduced limb growth (Perbal et al., 1996; Vincent et al., 2003; Efremova et al., 2001).
597 Petals are fused into a tube in snapdragon flowers, but the tube is much more reduced than in
598 petunia, hence conclusions on tube length restoration in the chimeras were not drawn by the
599 authors. However, in light of our results, it is clear that snapdragon chimeras expressing *DEF* or
600 *GLO* in the L2/L3 layers restore tube development to a higher degree than limb development,
601 similar to what we observed. In Arabidopsis that has simple and unfused petals, petal size was never
602 fully restored when *AP3* was expressed in one cell layer only, while petal shape was normal (Jenik
603 and Irish, 2001; Urbanus et al., 2010b); in contrast epidermal expression of *PI* was sufficient to
604 restore normal petal development (Bouhidel and Irish, 1996). Therefore, it seems that the
605 contribution of different cell layers to petal development varies across species and depending on the
606 petal identity gene under investigation.

607

608 **Autonomous and non-autonomous effects of *PhDEF* expression on petal traits**

609 Our study revealed that petal traits are affected differently by layer-specific *PhDEF* expression
610 (Supplemental Figure S6). For instance, epidermal pigmentation is a clearly autonomous trait, since
611 *star* petals are not pigmented except when wt revertant sectors arise. On the contrary, epidermal cell
612 shape appears to behave as a partially autonomous trait since *star* epidermal cells have an
613 intermediate phenotype between wt petal conical cells and sepal epidermal cells. Finally, organ size
614 and shape are specified non-autonomously in sub-domains of the petal: *PhDEF* expression in the L1
615 or L2 is sufficient to specify correct shape of the limb or correct size and shape of the tube
616 respectively, suggesting that in these petal domains, layer-specific *PhDEF* expression is sufficient
617 to signal cells from the other layer to grow normally.

618 The mechanisms for this inter-layer communication remain unknown. Our *in situ*
619 hybridization assays show that the *PhDEF* mRNA is not mobile between layers, but our attempts to
620 detect the PhDEF protein in petal tissue by immuno-histochemistry have been unsuccessful,
621 therefore we do not know if the PhDEF protein itself might be moving between layers, which would
622 be the simplest mechanistic explanation for the non-autonomous traits that we observe. Indeed, in
623 *Antirrhinum* petals expressing *DEF* in the L2/L3 layers, the DEF protein was found in small
624 amounts in the epidermis and it is likely why petals from these chimeras are faintly pigmented
625 (Perbal et al., 1996; Vincent et al., 2003). This indirectly suggests that no such movement occurs in
626 the *star* petals that are mostly white. In contrast, *Arabidopsis* AP3 and PI GFP-fusion proteins are
627 unable to move between cell layers, although they can move within the epidermal layer (Urbanus et
628 al., 2010a, 2010b). In any case, even if the PhDEF protein would move between layers in our
629 chimeric flowers, it is likely to be in small amounts only, and possibly at restricted stages of
630 development, otherwise both flower types would have a wt phenotype. Therefore, it is unlikely to
631 be the sole reason for tube and limb correct development in the *star* and *wico* flowers.

632 Alternatively, the non-autonomous effects that we observed might be triggered by
633 mechanical signals transmitted between layers. For instance, in *star* flowers normal growth of the
634 mesophyll could merely drag along epidermal cells, since cells are connected by their cell walls,
635 which could be sufficient to trigger their expansion and division. Other features, like conical cell
636 shape, might be directly influenced by mechanical signals. Indeed, conical cells are shaped by a
637 circumferential microtubule arrangement controlled by the microtubule-severing protein
638 KATANIN, and altering this arrangement affects conical cell shape (Ren et al., 2017). Microtubule
639 arrangement responds to mechanical signals (Hamant et al., 2008), which are likely to be

640 transmitted between layers. Therefore, it is possible that the formation of bulging cells in the *star*
641 epidermis is merely triggered by mechanical signals from the growing underlying layer,
642 independent of any petal identity specifier, as was recently evidenced from the observation of
643 conical-like bulges on the hypocotyl of the tubulin kinase mutant *nek6* (Takatani et al., 2020). The
644 molecular or physical nature of the signals involved in communication between layers remains to be
645 explored in full depth.

646

647 **Towards the gene regulatory networks of petal development**

648 Our *star* and *wico* material granted the opportunity to explore the gene regulatory networks driving
649 petal development in petunia, more specifically by decoupling on the one hand tube vs. limb
650 development, and epidermis vs. mesophyll development on the other. However, these effects are
651 confounded in our dataset, since both epidermis and limb development are affected in *star* flowers,
652 whereas both mesophyll and tube development are affected in *wico* flowers. Further analyses, such
653 as sequencing the transcriptome from *star* and *wico* limb and tube tissues separately, would help
654 uncouple these effects, but it is not easy to clearly separate these different domains during early
655 stages of development, which are crucial stages for petal morphogenesis. Spatial transcriptomics
656 techniques, such as single-cell RNA-Seq, would be ideal to precisely dissect transcriptional changes
657 between layers and domains of the petal at young developmental stages.

658 Still, we exploited our transcriptomic dataset by focusing our analysis on anthocyanin-
659 related genes, because the molecular link between the early establishment of petal identity by
660 homeotic transcription factors, such as PhDEF, and the late establishment of petal maturation traits,
661 such as anthocyanin accumulation, was unknown. For this, we examined the presence of
662 anthocyanin-related genes among genes downregulated both in *star* and *phdef-151* samples, but not
663 differentially expressed in *wico* samples. We found a very strong enrichment of anthocyanin-related
664 genes in this dataset, suggesting that the initial triggering event for most of the anthocyanin
665 biosynthesis pathway was missing in *star* flowers.

666 Finally, we investigated the direct link between PhDEF and petal pigmentation and found
667 that, *in vitro*, the PhDEF + PhGLO1 protein complex directly binds to predicted binding sites in the
668 regulatory regions of *AN1* and *AN2*. We confirmed that PhDEF binds to the corresponding genomic
669 region of *AN2* *in planta* by ChIP, but not for *AN1*, confirming that *in vitro* binding does not
670 necessarily imply *in vivo* binding, the last being strongly influenced by the local chromatin
671 landscape. The binding site of PhDEF that we identified on *AN2* (*AN2-bs3*) lies in the terminator

672 region of the gene (and the next gene on the chromosome is more than 100 kb away), which was
673 surprising since around 80 % of MADS binding sites are located within the 3 kb promoter region of
674 their target genes (Aerts et al., 2018). However, the presence of a binding site in the terminator
675 region is still compatible with an activating role in transcription, through DNA looping to the
676 promoter (Jash et al., 2012) or by promoting transcription termination and reinitiation (Wang et al.,
677 2000). Other putative CARG boxes in the genomic region of *AN2* are *AN2-bs1*, located 866 bp
678 upstream the ATG in the promoter region, and *AN2-bs2*, located 62 bp downstream the STOP codon
679 in the 3'UTR region. Both have non-canonical CARG box sequences (GAAAAGTAG for *AN2-bs1*
680 and TCTTTTTTAA for *AN2-bs2*) and were not bound in our gel shift assay (Supplemental Figure
681 S8). Still, it is possible that regulators other than MADS-box TFs could form protein complexes
682 with PhDEF and mediate looping to the promoter region of *AN2*. The precise mechanism by which
683 PhDEF might activate *AN2* transcription remains to be uncovered.

684 When aligning *Petunia AN2* sequences, we found that *AN2-bs3* lies in a globally non-
685 conserved region of the gene (Supplemental Figure S8), and *AN2-bs3* is only conserved in *Petunia*
686 *inflata*, one of the likely original parents of *Petunia x hybrida* (Bombarely et al., 2016). However,
687 *cis*-regulatory elements are very fluid and their sequences can change rapidly in short evolutionary
688 times, without the gene regulation being necessarily lost (see for instance (Schmidt et al., 2010;
689 Krieger et al., 2022)). Moreover, petal pigmentation is an extremely labile trait, and even within the
690 *Petunia* genus it has been lost and regained at least two times independently (Quattrocchio et al.,
691 1999; Esfeld et al., 2018; Berardi et al., 2021). Therefore, the fact that *AN2-bs3* is not largely
692 conserved does not necessarily imply that it is an unimportant site for *AN2* regulation in *P. hybrida*.

693 The fact that we detected strong *in planta* binding of PhDEF to *AN2*, together with the fact
694 that *AN2* expression is strongly down-regulated in the *phdef-151* transcriptome, suggests that
695 PhDEF is a good candidate to directly activate *AN2* expression in the petal. Ectopic expression of
696 *AN2* in *petunia* leaves is sufficient to trigger anthocyanin accumulation in this tissue, by inducing
697 *AN1* expression among others (Spelt et al., 2000; Quattrocchio et al., 1998). Therefore, if PhDEF
698 indeed activates *AN2* expression, it should be sufficient to launch the whole pigmentation pathway
699 in the wt petal limb. However, to fully support this conclusion, functional tests on the role of
700 PhDEF binding to *AN2-bs3* in regulating *AN2* expression should be conducted. A direct link
701 between petal identity and pigmentation has yet to be established, although genetic evidence in
702 orchid flowers strongly implied that different B-class proteins heteromeric complexes are
703 responsible for specific pigmentation spots in the different petal types, but physical binding of these

704 B-class protein complexes on pigmentation genes was not tested (Hsu et al., 2021). The direct target
705 genes of B-class proteins have been identified by ChIP-Seq and transcriptomic analyses in
706 Arabidopsis (Wuest et al., 2012), but this species has unpigmented petals, thereby preventing us to
707 draw any possible link between petal identity and pigmentation. The petunia petal is the ideal
708 system to test this direct link, and our results suggest that PhDEF might be the direct link between
709 petal identity and its epidermal pigmentation.

ACCEPTED MANUSCRIPT

710 MATERIALS AND METHODS

711 Plant materials, growth conditions and plant phenotyping

712 The *phdef-151* plants were obtained from the *Petunia x hybrida* W138 line and were grown in a
713 culture room in long day conditions (16h light 22°C; 8h dark 18°C; 75-WValoya NS12 LED bars;
714 light intensity: 130 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Hundreds of *phdef-151* flowers were observed over several years,
715 and all of them show the same phenotype, also identical to the *def-1* and *green petal (gp)* mutant
716 flowers (de Vlaming et al., 1984; van der Krol et al., 1993). The *wico* and *star* flowers were
717 repeatedly obtained from several different *phdef-151* individuals and were maintained by cuttings.
718 For this, branches where several *star* or *wico* flowers were already visible were cut into a ca. 5-cm
719 long segment, large flowers and leaves were removed and the branch segment was planted into an
720 hydrated Jiffy peat soil pellet (Jiffy Products International AS, Norway). When roots became visible
721 on the outside of the pellet, it was transferred into soil. Plant and flower pictures were obtained with
722 a CANON EOS 450D camera equipped with objectives SIGMA 18-50mm or SIGMA 50mm. To
723 measure tube length, the flower was cut longitudinally and photographed from the side. To measure
724 limb area, the limbs were flattened as much as possible on a glass slide covered with transparent
725 tape and photographed from the top. The photographs were used to measure D1 and D2 lengths and
726 limb area with ImageJ.

727 Genotyping

728 Extraction of genomic DNA from young leaf tissue was performed according to (Edwards et al.,
729 1991). The region spanning the *dTph1* insertion site in *PhDEF* was amplified using primers
730 MLY0935/MLY0936 (Supplemental Table S2). PCR products were separated on a 2% (w/v)
731 agarose gel, fragments of interest were purified using the NucleoSpin® Gel and PCR Clean-up kit
732 (Macherey-Nagel), and sequenced with Eurofins SupremeRun reactions.

733 *In situ* RNA hybridization

734 Floral buds from wt, 2 *wico* and 1 *star* lines were fixed overnight in FAA (3.7% (v/v) formaldehyde,
735 5% (v/v) acetic acid, 50% (v/v) ethanol), cleared in Histo-clear and embedded in paraffin to
736 perform 8 μm sections. *PhDEF* cDNA sequence was amplified from wt petunia inflorescence
737 cDNAs with primers MLY1738/MLY1739 (Supplementary Table 2), generating a 507 bp fragment
738 excluding the part encoding the highly conserved DNA-binding domain. The digoxigenin-labeled
739 RNA probe was synthesized from the PCR fragment by *in vitro* transcription, using T7 RNA
740 polymerase (Boehringer Mannheim). RNA transcripts were hydrolyzed partially for 42 min by
741 incubation at 60°C in 0.1 M $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ buffer, pH 10.2. Later steps were performed as

742 described by (Cañas et al., 1994). For imaging, slides were mounted in Entellan (Sigma) and
743 imaged with a Zeiss Axio Imager M2 light microscope equipped with a Zeiss Axio Cam HRc
744 camera.

745 **Petal cross-sections**

746 Small pieces (around 5 mm²) of tissue were harvested from the proximal and distal parts of wt
747 mature sepals, and from the tube and limbs of wt, *star* and *wico* mature petals. Samples were fixed
748 overnight in FAA (3.7% (v/v) formaldehyde, 5% (v/v) acetic acid, 50% (v/v) ethanol) and
749 dehydrated in an ethanol series. Preinfiltration was performed in a 1:1 mixture of ethanol:Technovit
750 7100 (Electron Microscopy Sciences) for 4 h under light agitation, then overnight in a 1:3
751 ethanol:Technovit 7100 mixture. Infiltration was performed in the infiltration solution for 1.5 h
752 under vacuum, then for one night followed by one additional week. Samples were arranged in the
753 moulds with the polymerization solution for 2 h at room temperature, then mounted with the
754 Technovit 3040 resin to relieve the blocks from the moulds. Blocks were sectioned with a
755 microtome to generate 3-7 µm-thick sections. Slides were incubated for 10 minutes in a 0.1% (w/v)
756 toluidine blue solution and imaged with a Zeiss Axio Imager M2 light microscope equipped with a
757 Zeiss Axio Cam HRc camera.

758 **Scanning electron microscopy (SEM)**

759 Scanning electron micrographs were obtained with a HIROX SH-1500 bench top environmental
760 scanning electron microscope equipped with a cooling stage. Samples were collected and quickly
761 imaged to limit dehydration, at -5°C and 5 kV settings. For cell area and length measurements,
762 pictures were taken from 3 petal tubes and 3 petal limbs from different wt, *star* and *wico* flowers.
763 For each sample, 3 pictures were taken and 5 cells (for the tube) or 10 cells (for the limb) were
764 measured for each picture. Measures were performed with ImageJ by manually drawing the outline
765 or length of the cells.

766 **RNA-Seq**

767 Petal tissue was collected at 1 pm from several plants stemming from a single *star* line, a single
768 *wico* line, and several individual wt plants (progeny of a single *star* flower) and *phdef-151* plants
769 (progeny of the same *star* flower). Tube length was macroscopically measured to compare stages,
770 the corolla was cut open and stamens were removed as much as possible from the corolla by pulling
771 on the filaments fused to the tube. One biological replicate contains total petal tissue from 2
772 flowers. Tissue was ground in liquid nitrogen and RNA was extracted with the Spectrum Plant Total
773 RNA Kit (Sigma) including on-column DNase digestion (Sigma). RNA integrity and quantity were

774 determined by a Bioanalyzer RNA 6000 Nano assay (Agilent). Libraries were prepared with poly-A
775 enrichment and single-end 75-bp sequencing was performed on a NextSeq 500 platform (Illumina).
776 16 to 23 million reads were recovered per library. Reads were checked for quality with FastQC
777 v0.11.4 (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>), adaptors and low-quality
778 ends were trimmed with Cutadapt v 1.16 (Martin, 2011) and custom Perl scripts. The reference
779 genome sequence used for transcriptome analysis is the *Petunia axillaris* v1.6.2 HiC genome
780 published in (Bombarely et al., 2016) and further scaffolded by HiC by DNAAZoo (Dudchenko et al.,
781 2017, 2018); gene annotations were transferred from the published assembly to the HiC-scaffolded
782 version using Blat (Kent, 2002), Exonerate (Slater and Birney, 2005) and custom Perl scripts. In the
783 rare cases when gene annotations from the published genome mapped to several regions in the HiC-
784 scaffolded genome, these different putative genes were identified by a letter added at the end of the
785 gene identifier (for instance Peaxi162Scf00179g00121a). The complete set of reads was mapped on
786 the reference genome sequence using HISAT2 v2.2.1 (Kim et al., 2015) to identify splicing sites,
787 before performing mapping sample per sample. Reads per gene were counted using FeatureCounts
788 v1.5.1 (Liao et al., 2014). DESeq2 version 3.12 (Love et al., 2014) was used with R version 4.0.3 to
789 perform the Principal Component Analysis and the differential gene expression analysis. Genes
790 having less than 10 reads in the sum of all samples were considered as non-expressed and
791 discarded. Genes were considered to be differentially expressed if $\log_2\text{FoldChange} > 1$ or < -1 , and
792 p-adjusted value < 0.01 . The bioinformatic pipeline for annotation transfer, read cleaning, splicing
793 site discovery, read mapping and preliminary DESeq2 results can be found at [gitbio.ens-](https://gitbio.ens-lyon.fr/rdp/petunia_star_wico_rnaseq)
794 [lyon.fr/rdp/petunia_star_wico_rnaseq](https://gitbio.ens-lyon.fr/rdp/petunia_star_wico_rnaseq). Venn diagrams were built with InteractiVenn (Heberle et al.,
795 2015). Due to the automatic gene name annotation pipeline used in (Bombarely et al., 2016) based
796 on homology with tomato (*Solanum lycopersicum*) proteins, many of the previously characterized
797 petunia genes have not been annotated according to their first described name, making
798 interpretation of some of the RNA-Seq results less straightforward. We have manually added
799 annotations of 42 genes from the anthocyanin biosynthesis pathway based on the Supplementary
800 Note 7 from (Bombarely et al., 2016), and 31 type-II MIKC-C MADS-box genes based on previous
801 studies from the literature ; these annotations can be found in the Supplemental Dataset S1 of this
802 manuscript. We noticed that the gene annotations from three major pigmentation genes, *DFR*
803 (*DIHYDROFLAVONOL-4-REDUCTASE*, Peaxi162Scf00366g00630), *CHS*a (*CHALCONE*
804 *SYNTHASE a*, Peaxi162Scf00047g01225) and *PHI* (Peaxi162Scf00569g00024) were lost during
805 the gene annotation transfer procedure, because they lie in regions of the genome that are still

806 poorly resolved. Therefore, we manually searched the position of these transcripts in the HISAT2
807 output and we were able to map part of the *DFR* and *CHSa* genes to two small scaffolds, while *PHI*
808 position was not found. We added the transcript positions of *DFR* and *CHSa* in the gtf/gff files
809 before running FeatureCounts. The read counts for *DFR* and *CHSa* reported in Supplemental Figure
810 S7 are therefore an under-estimation of their actual expression levels, since we miss part of the
811 genes.

812 **Prediction of MADS-box TF binding sites**

813 Genomic sequences from *AN1*, *AN2* and *PhDEF* from the *Petunia x hybrida* R27 line, starting 3 kb
814 upstream the START codon and ending 1 kb downstream the STOP codon, were scanned with all
815 MADS-box TF matrices included in the Jaspar 2020 database (<http://jaspar.genereg.net>), only
816 removing matrices from AGL42 and AGL55 which are much shorter than the other matrices and
817 therefore yield much higher scores. Relative scores above 0.86 were plotted against their genomic
818 position.

819 **Electrophoretic mobility shift assays (EMSAs)**

820 CDS sequences from *PhDEF* and *PhGLO1* were amplified from *Petunia x hybrida* R27
821 inflorescence cDNAs with primers MLY2382/MLY2383 and MLY2384/2385 respectively
822 (Supplemental Table S2) and cloned into the *in vitro* translation vector pSPUTK (Stratagene) by
823 NcoI/XbaI restriction. From these vectors, the PhDEF and PhGLO1 proteins were produced with
824 the TnT SP6 High-Yield Wheat Germ Protein Expression System (Promega) according to the
825 manufacturer's instructions. The terminator regions from *AN1* (0.8 kb) and *AN2* (1 kb), and part of
826 the promoter region of *AN2* (1.2 kb), were amplified from *Petunia x hybrida* R27 genomic DNA
827 with primers from Supplemental Table S2 and cloned into pCR-BluntII-TOPO (ThermoFisher).
828 Binding sites were amplified from these plasmids with primers listed in Supplemental Table 2, with
829 the forward primer labelled with Cy5 in 5'. The labelled DNA was purified and incubated with the
830 TnT *in vitro* translation mixture as described in (Silva et al., 2015) before loading on a native
831 acrylamide gel.

832 **PhDEF protein and antibody production**

833 The *PhDEF* truncated cDNA (without the sequence coding for the MADS domain) was chemically-
834 synthesized with optimization for expression in *Escherichia coli* and cloned into a pT7 expression
835 vector by Proteogenix (www.proteogenix.science). The expected PhDEF protein starts at amino
836 acid 60 (PSITT...) and ends at the last amino acid of the sequence (...FALLE), and a 6xHis tag was
837 added at the N-terminal part of the protein. The 6xHis-PhDEF protein was purified by affinity

838 column with a Nickel resin under denaturing conditions (8M urea) by Proteogenix. The purified
839 protein was injected in two rabbits for immunization by Proteogenix, to generate PhDEF-directed
840 polyclonal antibodies, that were purified by affinity against the antigen. Both lots of purified
841 antibodies were validated by immunoblot in petal or sepal tissues from wt, *phdef-151* and *phtm6*
842 samples.

843 **Chromatin immunoprecipitation (ChIP)**

844 One biological replicate comprises the full corolla from 2 flowers (wt), second whorl sepals from 3
845 flowers (*phdef-151*) or second whorl sepals from 3 to 4 flowers (*phglo1 phglo2*), and the full
846 experiment was performed for 3 biological replicates for wt and *phdef-151* and 2 biological
847 replicates for *phglo1 phglo2*. Samples at stage 8 were collected and ground in liquid nitrogen.
848 Ground tissue was resuspended into 10 mL fixation buffer (10 mM Hepes pH7.6, 0.5 M sucrose, 5
849 mM KCl, 5 mM MgCl₂, 5 mM EDTA pH8, Complete Protease Inhibitor Cocktail (Merck), 14 mM
850 2-mercaptoethanol) and a double cross-linking was performed at room temperature (1 hour with
851 disuccinimidyl glutarate at 2.5 mM with gentle shaking, and 5 minutes with formaldehyde 1%
852 (v/v)). Cross-linking was stopped by adding glycine at 200 mM and samples were put directly on
853 ice. Cells were lysed with a 40 mL-Dounce tissue grinder (Duran Wheaton Kimble), Triton X-100
854 was added at 0.6% (w/v) and the lysate was filtered subsequently through 100 µm and 40 µm nylon
855 meshes to recover nuclei. Nuclei were pelleted for 10 minutes at 3,000 g at 4°C, and the pellet was
856 resuspended in 300 µL of cold nuclear isolation buffer (i.e. fixation buffer without 2-
857 mercaptoethanol), carefully deposited on 600 µL of a 15% Percoll solution (15 % (v/v) Percoll, 10
858 mM Hepes pH8, 0.5 M sucrose, 5 mM KCl, 5 mM MgCl₂, 5 mM EDTA pH8) and centrifuged for 5
859 minutes at 2,000 g at 4°C. The pellet was resuspended into 900 µL of cold nuclear lysis buffer (50
860 mM Tris-HCl pH7.5, 0.1% (w/v) SDS, 10 mM EDTA pH8) to lyse the nuclei, and chromatin was
861 sonicated twice for 15 minutes with a Covaris S220 sonicator (peak power 105, Duty factor 5,
862 Cycles/Burst 200 for 900s). For each sample, 25 µL of magnetic protein-A Dynabeads and 25 µL of
863 magnetic protein-G Dynabeads (Invitrogen) were washed twice with 100 µL of cold ChIP dilution
864 buffer (15 mM Tris-HCl pH7.5, 150 mM NaCl, 1% (w/v) Triton X-100, 1 mM EDTA pH8) using a
865 magnetic rack (MagRack 6, Cytiva). Beads were mixed with 2.5 µg of anti-PhDEF antibody and 1.8
866 mL of cold ChIP dilution buffer, and incubated for 2 hours at 4°C on a rotating wheel. Sonicated
867 chromatin was centrifuged for 5 minutes at 15,000 g at 15°C, and 25 µL of supernatant (for wt
868 samples) or 50 µL of supernatant (for *phdef-151* and *phglo1 phglo2* samples) was added to the mix
869 of beads and antibody, and incubated overnight at 4°C on a rotating wheel. Beads were washed

870 twice (one quick wash and one long wash with 15 minutes incubation on a rotating wheel) with
871 each of the following buffers: low salt wash buffer (0.1% (w/v) SDS, 1% (w/v) Triton X-100, 2 mM
872 EDTA pH8, 20 mM Tris-HCl pH8, 150 mM NaCl), high salt wash buffer (0.1% (w/v) SDS, 1%
873 (w/v) Triton X-100, 2 mM EDTA pH8, 20 mM Tris-HCl pH8, 500 mM NaCl), LiCl wash buffer
874 (0.25 M LiCl, 1% (v/v) NP40/Igepal, 1% (w/v) deoxycholate, 1 mM EDTA pH8, 20 mM Tris-HCl
875 pH8) and TE buffer. Elution was performed twice with 250 μ L of elution buffer (0.1 M NaHCO₃,
876 1% (w/v) SDS) at 65°C. IP and input samples were decrosslinked overnight at 65°C by adding
877 NaCl at 200 mM, then incubating for 2 h at 42°C with 20 μ g proteinase K in 10 mM EDTA pH8
878 and 40 mM Tris-HCl pH6.5. DNA was purified with phenol:chloroform:isoamyl alcohol (25:24:1)
879 followed by chloroform:isoamyl alcohol (24:1), precipitated with ethanol at -20°C and the pellet
880 was washed with ethanol 70 %. The dry pellet was recovered in 50 μ L TE and 1 μ L was used for
881 each qPCR reaction, which was performed in technical triplicates for each biological replicate (3 for
882 wt and *phdef-151*, 2 for *phglo1 phglo2* and the control without antibody). The qPCR reaction was
883 performed with 1X FastStart Universal SYBR Green (Merck) and 0.3 μ M primer mix
884 (Supplemental Table S2), for 40 cycles (15 seconds at 95°C, 1 minute at 60°C) in a QuantStudio 6
885 Flex instrument (ThermoFisher). Percentage of input (enrichment) was calculated as $100 * e^{-(CtIN - \log_2(DF) - CtIP)}$, with e the efficiency of the primer pair, CtIN the average Ct value for the Input
886 sample, DF the dilution factor and CtIP the average Ct value for the IP sample), as described in
887 (Solomon et al., 2021). The significance of the enrichment was evaluated with a one-tailed t-test
888 comparing the enrichment of the test region to the average of the enrichments of the two negative
889 regions.

891 **Sequence alignments**

892 The genomic sequences (3 kb upstream of the transcription starting site, 1 kb downstream of the
893 STOP codon) of *AN2* from Solanaceae species were retrieved by blasting the *P. hybrida AN2* coding
894 sequence against genomic sequence resources: *AN2* sequences from *Nicotiana tabacum* (K326)
895 (Sierro et al., 2014), *Petunia axillaris* and *Petunia inflata* (Bombarely et al., 2016) were retrieved
896 from the Sol Genomics Network website (solgenomics.net); *AN2* sequence from *Petunia exserta*
897 was retrieved from DNA Zoo (https://www.dnazoo.org/assemblies/Petunia_exserta); *AN2* sequence
898 from *Petunia secreta* was retrieved from NCBI GenBank, BioProject PRJNA674325. *AN2* genomic
899 sequences were aligned using mVista (Mayor et al., 2000) with *P. hybrida AN2* as reference, with
900 the AVID algorithm. Detailed alignment of the *AN2-bs3* region was performed with KAlign
901 (Lassmann, 2019) and visualized with MView (Madeira et al., 2022).

902

903 **Statistical analysis**

904 RStudio was used for statistical analysis of the numerical data. To test for differences in mean
905 values between samples, a Shapiro-Wilk test was performed to test for normal distribution of the
906 data, and accordingly to the results, either a Student's t-test or a Wilcoxon rank sum test was
907 applied. To test for differences between expected and observed frequencies, a Chi-square test or a
908 Fisher's exact test (for small samples) was applied. Details about the conditions used for the tests
909 are given in the corresponding Figure or Table legends, and all statistical test results are reported in
910 Supplemental Data Set S3.

911

912 **ACCESSION NUMBERS**

913 Sequence data from this article can be found in the EMBL/GenBank data libraries under accession
914 numbers OQ418981 (*AN1*), OQ418982 (*AN2*) and OQ418983 (*PhDEF*). Raw sequence reads for
915 the wt, *phdef-151*, *star* and *wico* second whorl organs transcriptome have been deposited in
916 BioProject with the accession number PRJNA951505.

917 **SUPPLEMENTAL DATA**

918 **Supplemental Figure S1.** Additional pictures of *star* and *wico* flowers.

919 **Supplemental Figure S2.** Stamens are unfused to the tube in *wico* flowers.

920 **Supplemental Figure S3.** Additional pictures of *PhDEF* transcript *in situ* hybridization in wild-
921 type, *star* and *wico* flowers.

922 **Supplemental Figure S4.** Wild-type and pink wild-type flowers observed in the progeny of a *star*
923 parent.

924 **Supplemental Figure S5.** Epidermal revertant sectors on *star* petals.

925 **Supplemental Figure S6.** Autonomous and non-autonomous effects in *star* and *wico* petals.

926 **Supplemental Figure S7.** Expression of B-class genes and a subset of pigmentation genes in wild-
927 type, *star*, *wico* and *phdef-151* samples.

928 **Supplemental Figure S8.** Additional information on putative PhDEF binding sites on the *AN2*
929 genomic sequence.

930

931 **Supplemental Table S1.** Genotyping results of the progeny of a *star* flower.

932 **Supplemental Table S2.** List of primers used in this study.

933

934 **Supplemental Dataset S1.** Differential gene expression calculated by DESeq2.

935 **Supplemental Dataset S2.** List of the 451 genes downregulated in *star* and *phdef-151* samples, and
936 not differentially expressed in *wico* samples.

937 **Supplemental Dataset S3.** Summary of statistical analyses.

938

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955

956 **AUTHOR CONTRIBUTIONS**

957 M.M. and M.V. conceived and designed the experiments. M.C., Q.C.S., P.M., P.C., V.H. and S.R.B.
958 performed the experiments. M.C., Q.C.S., J.J., M.V. and M.M. analyzed the data. M.C., C.Z., M.V.
959 and M.M. wrote the article.

960 **TABLES**961 **Table 1.** Progeny of the *star* and *wico* flowers after selfing.

962 7 *wico* flowers and 4 *star* flowers have been selfed and their progeny has been phenotyped and
 963 classified into *phdef*, wt or pink wt phenotype. Summing the *star* progeny for the 4 parents gives 25
 964 *phdef*, 16 wt and 39 pink wt plants, which is not significantly different to a 1:1:2 ratio (chi-square
 965 test, $p = 0.35$). * For *wico*, we found 4 plants with wt or pink wt flowers in the progeny, and all of
 966 them were linked to the presence of a *de novo* transposon excision from the *PhDEF* locus, restoring
 967 either a *PhDEF+6* (in the case of pink wt progeny) or a wild-type *PhDEF* (in the case of the wt
 968 progeny) allele.

969

		Phenotype of the progeny (% of the total)		
		<i>phdef</i>	wt	pink wt
Parent flower	<i>wico-1</i>	15 (94%)		1 (6%) *
	<i>wico-2</i>	14 (88%)	1 (6%) *	1 (6%) *
	<i>wico-3</i>	16 (100%)		
	<i>wico-4</i>	15 (94%)		1 (6%) *
	<i>wico-5</i>	16 (100%)		
	<i>wico-6</i>	12 (100%)		
	<i>wico-7</i>	12 (100%)		
	<i>star-1</i>	11 (46%)	4 (17%)	9 (38%)
	<i>star-2</i>	4 (25%)	4 (25%)	8 (50%)
	<i>star-3</i>	7 (29%)	5 (21%)	12 (50%)
	<i>star-4</i>	3 (19%)	3 (19%)	10 (63%)

970

971

972

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974

975 **FIGURE LEGENDS**

976 **Figure 1. Macroscopic description of the *star* and *wico* flowers.**

977 (A) *phdef-151* mutant plant harboring one branch with *wico* revertant flowers and one branch with
 978 *star* revertant flowers. Scale bar: 1 cm. (B-I) Representative wild-type (wt) (B), *phdef-151* (C), *star*
 979 (D-F) and *wico* (G-I) flowers from a top (left) and side (right) view. The *star* and *wico* flowers come
 980 from independent reversion events (from different *phdef-151* plants or from different branches of a
 981 single *phdef-151* plant). Scale bar: 1 cm. (J) Two *star* flowers with additional L1-revertant sectors
 982 in one petal (left) or one petal and two half petals (right). Scale bar: 1 cm. (K) Schematic cross-
 983 section of a wt flower, showing stamens (in green) partially fused to the petal tube. The region of
 984 the tube fused to stamens is named D1, and the region of the tube where stamens are free is named
 985 D2, as defined in (Stuurman et al., 2004). (L) Average length of regions D1, D2 and total tube
 986 length in wt, *star* and *wico* flowers. (M) Average limb area in wt, *star* and *wico* flowers. (N)
 987 Average ratio between limb area and tube length in wt, *star* and *wico* flowers. n = 7 wt flowers, n =
 988 12 *star* flowers from 4 different branches, n = 18 *wico* flowers from 5 different branches. Student's t
 989 test, two-sided with Welch correction for D1, D2 and tube length, two-sided without Welch
 990 correction for limb area and limb area/tube length ratio (* p < 0.05, ** p < 0.01, *** p < 0.005).
 991 Error bars represent \pm s.e.m.

992

993 **Figure 2. Sequencing the *PhDEF* excision alleles in *star* and *wico* flowers.**

994 (A) *PhDEF* gene model indicating the position of the *dTph1* insertion in the first exon (black
 995 triangle) and the primers used for subsequent amplification and sequencing (in red). (B) Amplicons
 996 generated with primers spanning the *dTph1* insertion site, on genomic DNA from *phdef-151* second
 997 whorl organs and *star* and *wico* sepals and petals. The large fragment still contains the *dTph1*
 998 transposon inserted (expected size: 407 bp), while small fragments result from different events of
 999 *dTph1* excision (expected size: 115 bp) and were subsequently sequenced. (C) The small *PhDEF*
 1000 fragments from (B) were sequenced in the second whorl organs of flowers with a *phdef* (n = 2), *star*
 1001 (n = 14) and *wico* (n = 14) phenotype. The nucleotidic sequence and predicted protein sequence are
 1002 indicated, with stop codons represented by a *star*. Additional nucleotides or amino-acids as
 1003 compared to the wt sequences are indicated in red. n = number of independent reversion events
 1004 where the same excision footprint was found. wt = wild-type.

1005

1006 **Figure 3. Localization of the *PhDEF* transcript in wt, *star* and *wico* flowers by *in situ***
 1007 **hybridization.**

1008 Longitudinal sections of wild-type (wt) (A, B, C), *star* (D, E, F) and *wico* (G, H, I) flowers or young
 1009 petals hybridized with a digoxigenin-labelled *PhDEF* antisense probe. At the earliest stage chosen
 1010 (A, D, G), sepals are initiating and *PhDEF* is expressed in the future petal / stamen initiation
 1011 domain. Note that if the section was not performed at the center of the flower, the *PhDEF* signal
 1012 might artificially appear to be in the middle of the flower (as in D) whereas it is actually on its
 1013 flanks. At the middle stage chosen (B, E, H), stamens (white arrowhead) and petals (red arrowhead)
 1014 are initiating, and *PhDEF* is expressed in both primordia. The meristematic L1, L2 and L3 layers
 1015 are indicated on the wt sections (A, B). *PhDEF* expression is also detected at the tip of young petal
 1016 limb (C, F, I). The epidermis and mesophyll layers, derived from the previous L1 and L2
 1017 meristematic layers, are indicated on the wt section (C). se: sepals. Scale bar: 50 μ m.

1018

1019 **Figure 4. Epidermal and mesophyll cell identities in wt petals and sepals, and *star* and *wico***
 1020 **petals.**

1021 **(A)** From left to right: wild-type (wt) petals, wt sepals, *star* petals and *wico* petals cut open
 1022 longitudinally to show areas used for scanning electron microscopy and cross-sections. Petals were
 1023 subdivided into limb and tube area, and sepals were subdivided into a distal and a proximal part, as
 1024 shown by the dotted white rectangles. Scale bar: 1 cm. **(B)** Representative scanning electron
 1025 micrographs from the adaxial side of a wt petal, wt sepal, *star* petal and *wico* petal (from left to
 1026 right). The red arrowhead points to a stomata and the white arrowhead points to a trichome. Scale
 1027 bar: 30 μ m. **(C)** Representative cross-sections from wt petals, wt sepals, *star* petals and *wico* petals
 1028 (from left to right) stained with toluidine blue. The adaxial and abaxial epidermis and the mesophyll
 1029 are indicated on the wt petal sections. Scale bar: 100 μ m. **(D)** Average limb cell area from the
 1030 adaxial side of wt, *star* and *wico* petals (n = 30 cells). Student's t test with Welch correction, two-
 1031 sided (* p < 0.05, ** p < 0.01, *** p < 0.005). Error bars represent \pm s.e.m. **(E)** Average tube cell
 1032 length from the adaxial side of wt, *star* and *wico* petals (n = 40 cells for wt, 45 cells for *star* and
 1033 *wico*). Wilcoxon rank sum test, two-sided (* p < 0.05, ** p < 0.01, *** p < 0.005). Error bars
 1034 represent \pm s.e.m. **(F)** Limb area from wt (top) and *wico* (bottom) petals, after their adaxial
 1035 epidermis was manually peeled. For wt, the upper half of the picture shows the white underlying
 1036 mesophyll. For *wico*, the green triangular area shows the green (chloroplastic) underlying
 1037 mesophyll. Scale bar: 300 μ m.

1038

1039 **Figure 5. Gene differential expression in *star* and *wico* petals.**

1040 (A) Flowers from wild-type (wt), *star*, *wico* and *phdef-151* at stages 4, 8 and 12 (only stage 12 for
 1041 *phdef-151*), whose petals or sepals were harvested for transcriptome sequencing. Flowers at anthesis
 1042 are shown for comparison. Scale bar: 1 cm. (B) Principal Component Analysis plot of the samples
 1043 after analysis of variance with DESeq2, showing that the first principal component corresponds to
 1044 the developmental stage and the second principal component corresponds to the genotype. (C)
 1045 Number of upregulated and downregulated genes in *star*, *wico* and *phdef-151*, as compared to wt at
 1046 the corresponding stages. (D) Venn diagram recapitulating the number of differentially expressed
 1047 genes (DEGs) in *star*, *wico* and *phdef-151* petal samples at stage 12, as compared to wt, and their
 1048 different intersections. Each sector contains the number of DEGs, and between parenthesis is the
 1049 percentage of genes that it represents from the total number of DEGs in the corresponding sample,
 1050 with a colour code (red = percentage of DEGs from *star* samples / blue = from *wico* samples / black
 1051 = from *phdef-151* samples).

1052

1053 **Figure 6. PhDEF binds to AN2 regulatory region *in vitro* and *in vivo*.**

1054 (A, B) Expression (as normalized read counts calculated by DESeq2) of *ANI* (A) and *AN2* (B) in
 1055 wild-type (wt), *star*, *wico* and *phdef-151* second whorl organs at stages 4, 8 or 12. Stars indicate
 1056 significant down-regulation ($\log_2FC < -1$ and adjusted p-value < 0.01). (C-E) Relative score
 1057 profiles for AP3 (red diamond), PI (blue triangle) and all other MADS-box transcription factors
 1058 (black dots) available on Jaspas, on the genomic sequences of *PhDEF* (C), *ANI* (D) and *AN2* (E).
 1059 The relative score is computed using the position weight matrix of each transcription factor and is
 1060 between 0 and 1; only relative scores higher than 0.86 are shown here. The gene model is
 1061 represented above the score profile with exons as grey rectangles, the transcription start site as an
 1062 arrow, and the gene model is aligned with the position of the predicted binding sites (bs). For
 1063 *PhDEF*, the position of a putative CArG box, as explained in the main text, is indicated by a red
 1064 arrow. The positions of the sites tested by gel shift in panel F and Supplemental Figure S8 are
 1065 indicated: putative PhDEF binding sites (*ANI-bs1*, *AN2-bs1*, *AN2-bs2* and *AN2-bs3*) and a negative
 1066 control with a low predicted binding score (*ANI-bs2*). Sites indicated in red were bound in the gel
 1067 shift assay, while sites indicated in grey were not bound. In orange, are depicted the genomic
 1068 fragments (GF) tested by chromatin immunoprecipitation in (G). (F) Representative electrophoretic
 1069 mobility shift assay (EMSA) gel performed with a combination of *in vitro*-translated PhDEF and/or

1070 PhGLO1 proteins, and Cy5-labelled *ANI-bs1*, *ANI-bs2* or *AN2-bs3* DNA fragments, whose position
1071 is depicted in (C-E). Similar results were obtained in 5 additional independent assays for *ANI-bs1*,
1072 2 additional independent assays for *AN2-bs3* and 4 additional independent assays for *ANI-bs2*. **(G)**
1073 Enrichment (as percentage of INPUT) of binding of PhDEF to different genomic regions of the
1074 chromatin purified from wt, *phdef-151* or *phglo1 phglo2* second whorl organs at stage 8, after
1075 immunoprecipitation with an anti-PhDEF directed antibody. The control without antibody was
1076 performed on chromatin isolated from wt petals. The position of the genomic fragments tested is
1077 depicted in (C-E). Neg1 and Neg2 represent two negative control fragments located in the promoter
1078 region of genes not differentially expressed in the *phdef-151* mutant, and present on different
1079 chromosomes than *PhDEF*, *ANI* and *AN2*. For unknown reasons, the Neg1 control region could
1080 never be amplified in the *phglo1 phglo2* samples. Stars indicate a significant enrichment of test
1081 regions over the average of the two negative control regions for each chromatin sample (one-sided
1082 t-test with Welch correction, * $p < 0.05$, ** $p < 0.005$; n = 3 biological replicates for wt and *phdef-151*,
1083 2 biological replicates for *phglo1 phglo2* and the control without antibody). Error bars represent \pm
1084 s.e.m.

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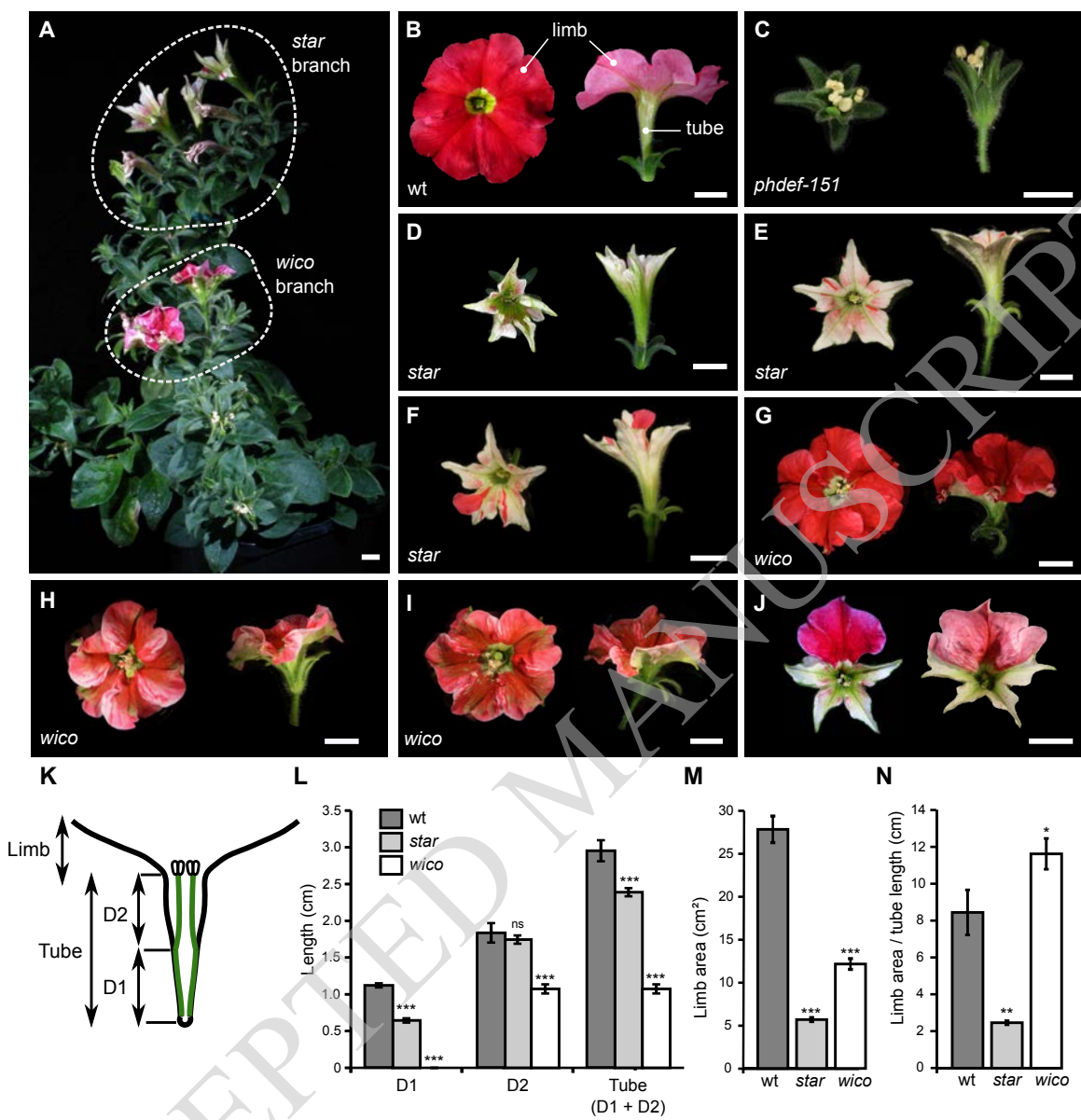
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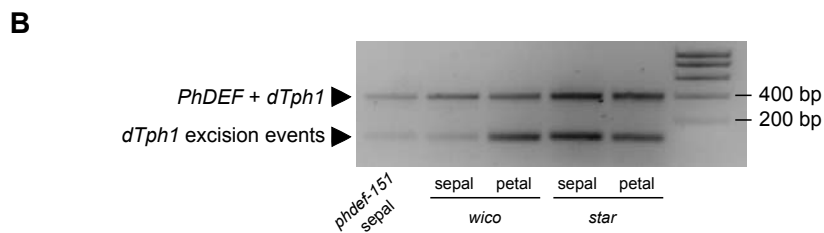
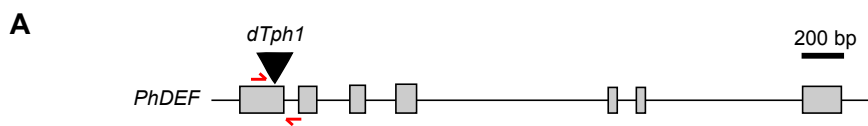


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Figure 1. Macroscopic description of the *star* and *wico* flowers.

(A) *phdef-151* mutant plant harboring one branch with *wico* revertant flowers and one branch with *star* revertant flowers. Scale bar: 1 cm. (B-I) Representative wild-type (wt) (B), *phdef-151* (C), *star* (D-F) and *wico* (G-I) flowers from a top (left) and side (right) view. The *star* and *wico* flowers come from independent reversion events (from different *phdef-151* plants or from different branches of a single *phdef-151* plant). Scale bar: 1 cm. (J) Two *star* flowers with additional L1-revertant sectors in one petal (left) or one petal and two half petals (right). Scale bar: 1 cm. (K) Schematic cross-section of a wt flower, showing stamens (in green) partially fused to the petal tube. The region of the tube fused to stamens is named D1, and the region of the tube where stamens are free is named D2, as defined in (Stuurman et al., 2004). (L) Average length of regions D1, D2 and total tube length in wt, *star* and *wico* flowers. (M) Average limb area in wt, *star* and *wico* flowers. (N) Average ratio between limb area and tube length in wt, *star* and *wico* flowers. n = 7 wt flowers, n = 12 *star* flowers from 4 different branches, n = 18 *wico* flowers from 5 different branches. Student's t test, two-sided with Welch correction for D1, D2 and tube length, two-sided without Welch correction for limb area and limb area/tube length ratio (* p < 0.05, ** p < 0.01, *** p < 0.005). Error bars represent \pm s.e.m.

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Phenotype	Allele	Nucleotidic sequence	Protein Sequence	n
wt	<i>PhDEF</i>	CCAGTA-----CTGG-----CAAGCTTCAT	DAKVSIIIMISS--TG--KLHEFIS	
<i>phdef</i>	<i>phdef (+8)</i>	CCAGTA-----CTGG CAGTCTGG CAAGCTTCAT	DAKVSIIIMISS--TG SLASFMNSL (+22aa) *	1
	<i>phdef (+7)</i>	CCAGTA-----CTGG C-GTCTGG CAAGCTTCAT	DAKVSIIIMISS--TG VWQAS *	1
<i>star</i>	<i>PhDEF</i>	CCAGTA-----CTGG-----CAAGCTTCAT	DAKVSIIIMISS--TG--KLHEFIS	1
	<i>phdef (+6a)</i>	CCAGTA-----CTGG-- GCTGG CAAGCTTCAT	DAKVSIIIMISS--TG SG KLHEFIS	7
	<i>phdef (+6b)</i>	CCAGTA-----CTGG CA-T-TGG CAAGCTTCAT	DAKVSIIIMISS--TG IG KLHEFIS	6
<i>wico</i>	<i>PhDEF</i>	CCAGTA-----CTGG-----CAAGCTTCAT	DAKVSIIIMISS--TG--KLHEFIS	3
	<i>phdef (+6a)</i>	CCAGTA-----CTGG-- GCTGG CAAGCTTCAT	DAKVSIIIMISS--TG SG KLHEFIS	7
	<i>phdef (+6c)</i>	CCAGTA-----CTGG CA-CTGG CAAGCTTCAT	DAKVSIIIMISS--TG TG KLHEFIS	3
	<i>phdef (+6d)</i>	CCAGTA GCCA GTCTGG-----CAAGCTTCAT	DAKVSIIIMISS SGS G--KLHEFIS	1

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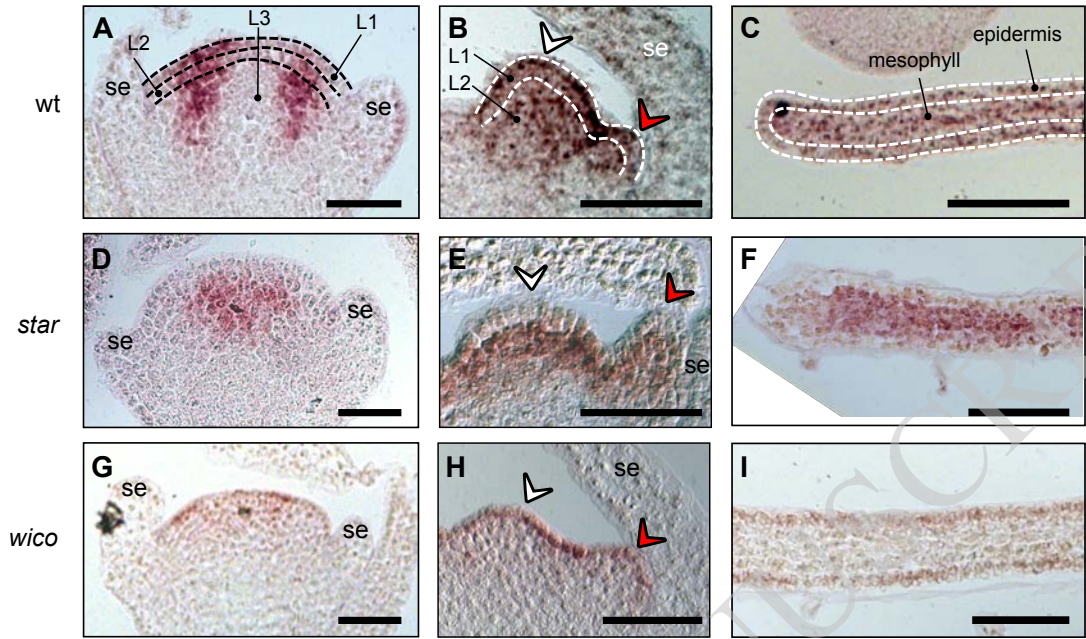
Figure 2. Sequencing the *PhDEF* excision alleles in *star* and *wico* flowers.

(A) *PhDEF* gene model indicating the position of the *dTph1* insertion in the first exon (black triangle) and the primers used for subsequent amplification and sequencing (in red). **(B)**

Amplicons generated with primers spanning the *dTph1* insertion site, on genomic DNA from *phdef-151* second whorl organs and *star* and *wico* sepals and petals. The large fragment still contains the *dTph1* transposon inserted (expected size: 407 bp), while small fragments result from different events of *dTph1* excision (expected size: 115 bp) and were subsequently sequenced. **(C)**

The small *PhDEF* fragments from (B) were sequenced in the second whorl organs of flowers with a *phdef* (n = 2), *star* (n = 14) and *wico* (n = 14) phenotype. The nucleotidic sequence and predicted protein sequence are indicated, with stop codons represented by a *star*. Additional nucleotides or amino-acids as compared to the wt sequences are indicated in red. n = number of independent reversion events where the same excision footprint was found. wt = wild-type.

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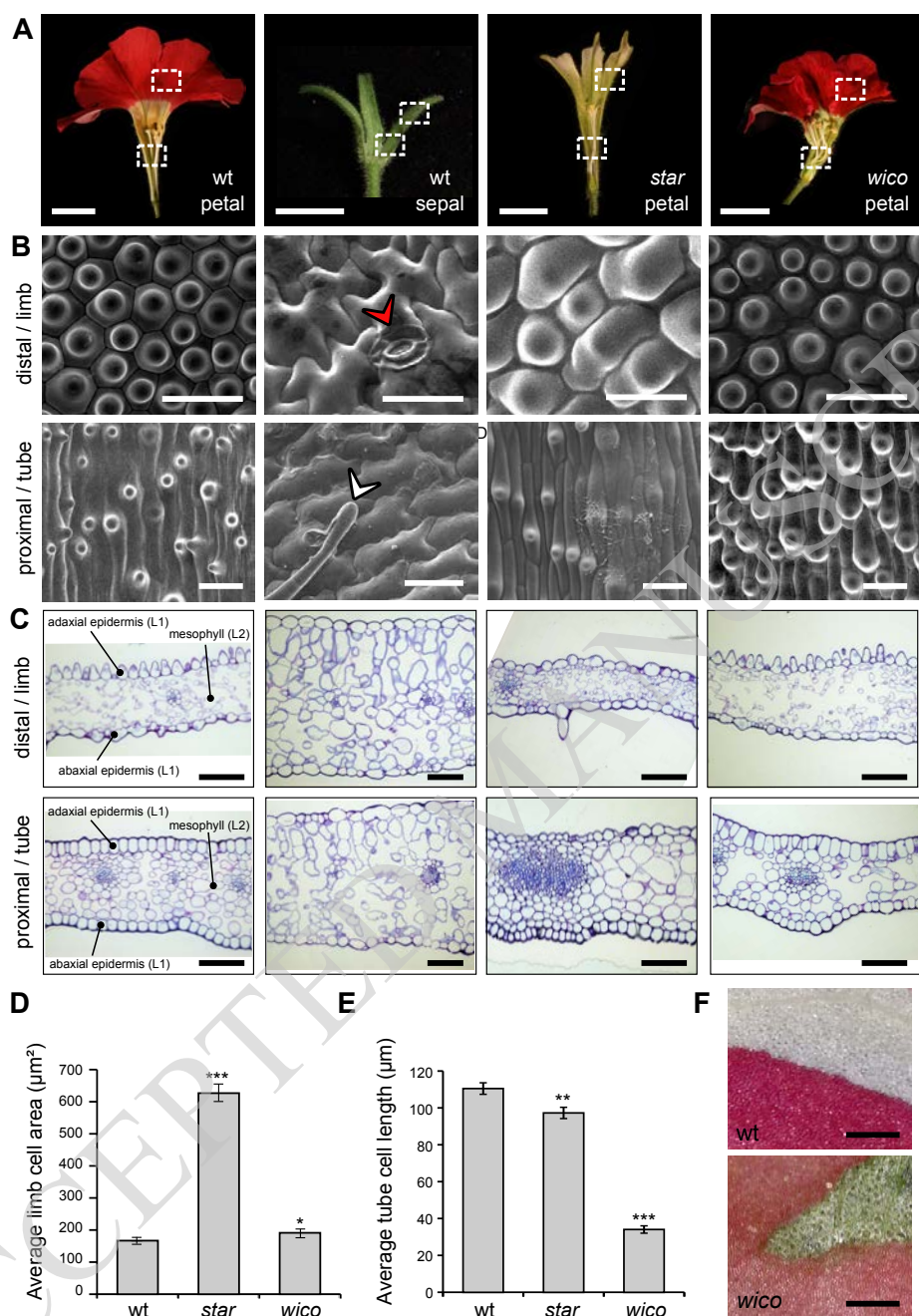


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Figure 3. Localization of the *PhDEF* transcript in wt, *star* and *wico* flowers by *in situ* hybridization.

Longitudinal sections of wild-type (wt) (A, B, C), *star* (D, E, F) and *wico* (G, H, I) flowers or young petals hybridized with a digoxigenin-labelled *PhDEF* antisense probe. At the earliest stage chosen (A, D, G), sepals are initiating and *PhDEF* is expressed in the future petal / stamen initiation domain. Note that if the section was not performed at the center of the flower, the *PhDEF* signal might artificially appear to be in the middle of the flower (as in D) whereas it is actually on its flanks. At the middle stage chosen (B, E, H), stamens (white arrowhead) and petals (red arrowhead) are initiating, and *PhDEF* is expressed in both primordia. The meristematic L1, L2 and L3 layers are indicated on the wt sections (A, B). *PhDEF* expression is also detected at the tip of young petal limb (C, F, I). The epidermis and mesophyll layers, derived from the previous L1 and L2 meristematic layers, are indicated on the wt section (C). se: sepals. Scale bar: 50 μ m.

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Figure 4. Epidermal and mesophyll cell identities in wt petals and sepals, and *star* and *wico* petals.

(A) From left to right: wild-type (wt) petals, wt sepals, *star* petals and *wico* petals cut open longitudinally to show areas used for scanning electron microscopy and cross-sections. Petals were subdivided into limb and tube area, and sepals were subdivided into a distal and a proximal part, as shown by the dotted white rectangles. Scale bar: 1 cm. **(B)** Representative scanning electron micrographs from the adaxial side of a wt petal, wt sepal, *star* petal and *wico* petal (from left to right). The red arrowhead points to a stomata and the white arrowhead points to a trichome. Scale bar: 30 μm . **(C)** Representative cross-sections from wt petals, wt sepals, *star* petals and *wico* petals (from left to right) stained with toluidine blue. The adaxial and abaxial epidermis and the mesophyll are indicated on the wt petal sections. Scale bar: 100 μm . **(D)** Average limb cell area from the adaxial side of wt, *star* and *wico* petals ($n = 30$ cells). Student's t test with Welch correction, two-sided (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$). Error bars represent \pm s.e.m. **(E)** Average tube cell length from the adaxial side of wt, *star* and *wico* petals ($n = 40$ cells for wt, 45 cells for *star* and *wico*). Wilcoxon rank sum test, two-sided (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$). Error bars represent \pm s.e.m. **(F)** Limb area from wt (top) and *wico* (bottom) petals, after their adaxial epidermis was manually peeled. For wt, the upper half of the picture shows the white underlying mesophyll. For *wico*, the green triangular area shows the green (chloroplastic) underlying mesophyll. Scale bar: 300 μm .

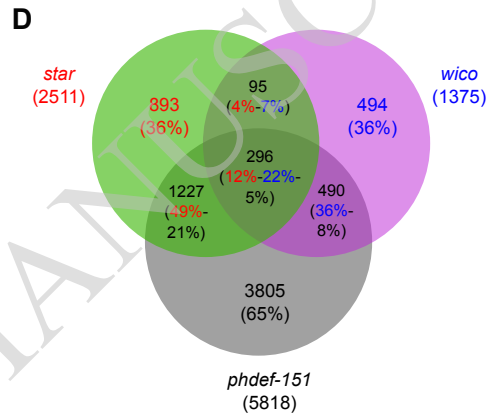
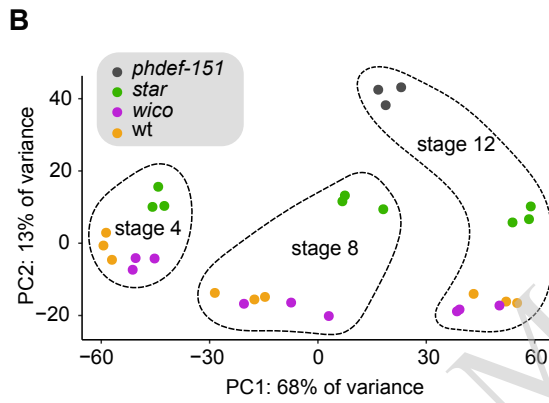
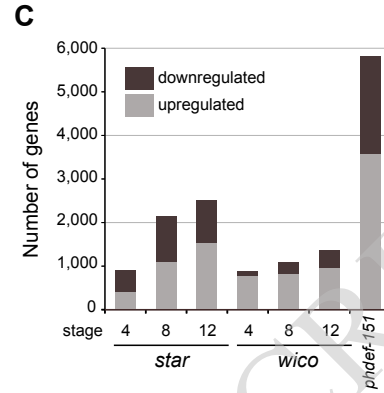
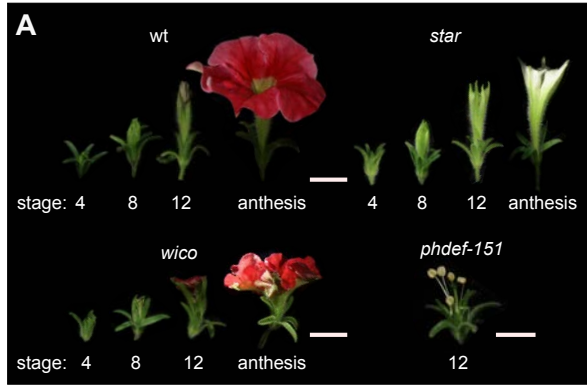
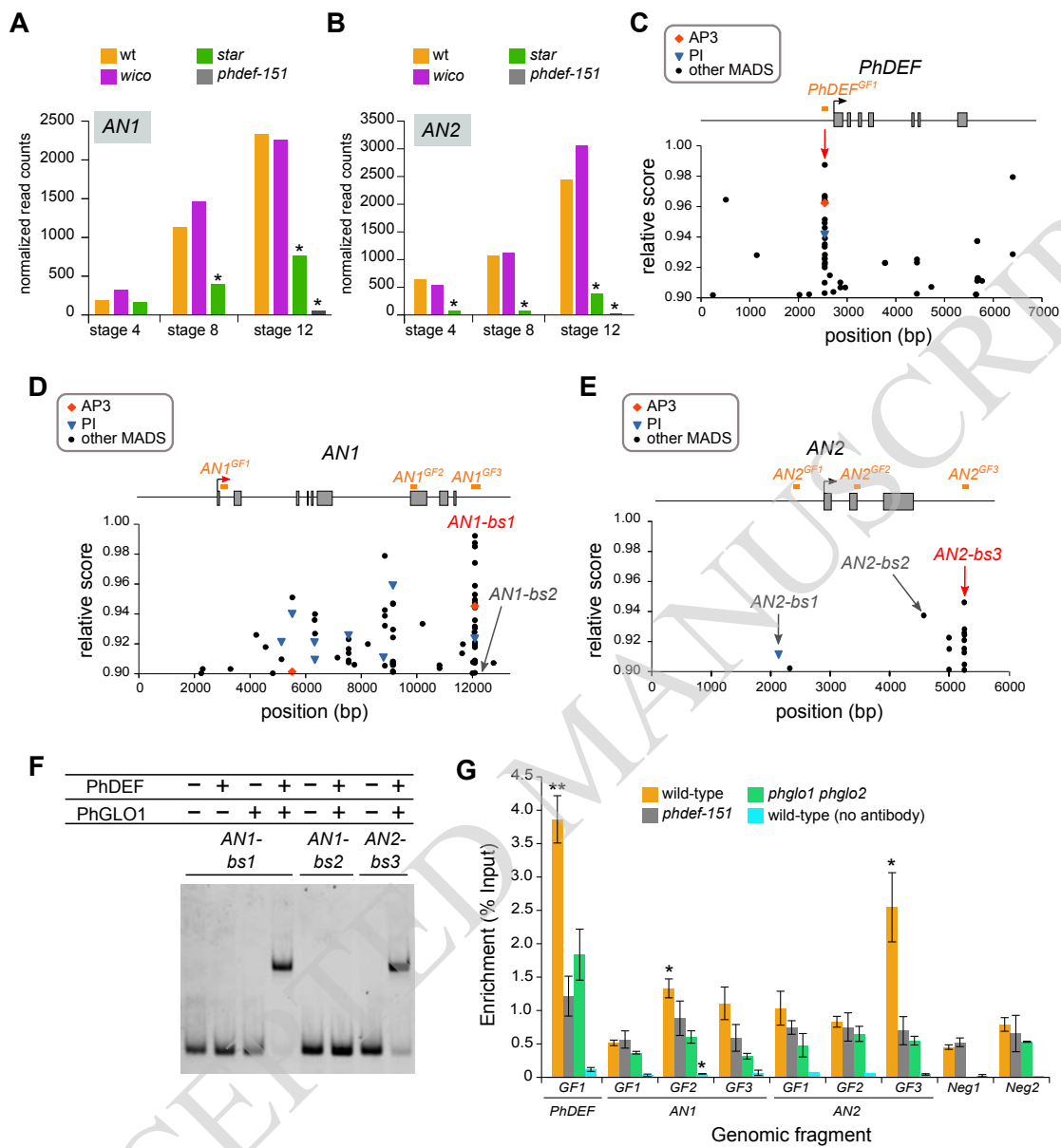


Figure 5. Gene differential expression in *star* and *wico* petals.

(A) Flowers from wild-type (wt), *star*, *wico* and *phdef-151* at stages 4, 8 and 12 (only stage 12 for *phdef-151*), whose petals or sepals were harvested for transcriptome sequencing. Flowers at anthesis are shown for comparison. Scale bar: 1 cm. (B) Principal Component Analysis plot of the samples after analysis of variance with DESeq2, showing that the first principal component corresponds to the developmental stage and the second principal component corresponds to the genotype. (C) Number of upregulated and downregulated genes in *star*, *wico* and *phdef-151*, as compared to wt at the corresponding stages. (D) Venn diagram recapitulating the number of differentially expressed genes (DEGs) in *star*, *wico* and *phdef-151* petal samples at stage 12, as compared to wt, and their different intersections. Each sector contains the number of DEGs, and between parenthesis is the percentage of genes that it represents from the total number of DEGs in the corresponding sample, with a colour code (red = percentage of DEGs from *star* samples / blue = from *wico* samples / black = from *phdef-151* samples).

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Figure 6. PhDEF binds to AN2 regulatory region *in vitro* and *in vivo*.

(A, B) Expression (as normalized read counts calculated by DESeq2) of *ANI* (A) and *AN2* (B) in wild-type (wt), *star*, *wico* and *phdef-151* second whorl organs at stages 4, 8 or 12. Stars indicate significant down-regulation ($\log_2FC < -1$ and adjusted p-value < 0.01). (C-E) Relative score profiles for AP3 (red diamond), PI (blue triangle) and all other MADS-box transcription factors (black dots) available on Jaspar, on the genomic sequences of *PhDEF* (C), *ANI* (D) and *AN2* (E). The relative score is computed using the position weight matrix of each transcription factor and is between 0 and 1; only relative scores higher than 0.86 are shown here. The gene model is represented above the score profile with exons as grey rectangles, the transcription start site as an arrow, and the gene model is aligned with the position of the predicted binding sites (bs). For *PhDEF*, the position of a putative CARG box, as explained in the main text, is indicated by a red arrow. The positions of the sites tested by gel shift in panel F and Supplemental Figure S8 are indicated: putative PhDEF binding sites (*ANI-bs1*, *AN2-bs1*, *AN2-bs2* and *AN2-bs3*) and a negative control with a low predicted binding score (*ANI-bs2*). Sites indicated in red were bound in the gel shift assay, while sites indicated in grey were not bound. In orange, are depicted the genomic fragments (GF) tested by chromatin immunoprecipitation in (G). (F) Representative electrophoretic mobility shift assay (EMSA) gel performed with a combination of *in vitro*-translated PhDEF and/or PhGLO1 proteins, and Cy5-labelled *ANI-bs1*, *ANI-bs2* or *AN2-bs3* DNA fragments, whose position is depicted in (C-E). Similar results were obtained in 5 additional independent assays for *ANI-bs1*, 2 additional independent assays for *AN2-bs3* and 4 additional independent assays for *ANI-bs2*. (G) Enrichment (as percentage of INPUT) of binding of PhDEF to different genomic regions of the chromatin purified from wt, *phdef-151* or *phglo1 phglo2* second whorl organs at stage 8, after immunoprecipitation with an anti-PhDEF directed antibody. The control without antibody was performed on chromatin isolated from wt petals. The position of the genomic fragments tested is depicted in (C-E). Neg1 and Neg2 represent two negative control fragments located in the promoter region of genes not differentially expressed in the *phdef-151* mutant, and present on different chromosomes than *PhDEF*, *ANI* and *AN2*. For unknown reasons, the Neg1 control region could never be amplified in the *phglo1 phglo2* samples. Stars indicate a significant enrichment of test regions over the average of the two negative control regions for each chromatin sample (one-sided t-test with Welch correction, * $p < 0.05$, ** $p < 0.005$; $n = 3$ biological replicates for wt and *phdef-151*, 2 biological replicates for *phglo1 phglo2* and the control without antibody). Error bars represent \pm s.e.m.

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