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

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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

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## 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

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## 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  $\gamma$ -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)<sub>6</sub>GG, 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 (CTATATTTGG) 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<sup>GF1</sup>* (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<sup>GF3</sup>* (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<sup>GF3</sup>*). 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  $\mu\text{m}$  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 mm<sup>2</sup>) 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), *CHSa* (*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](http://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 MgCl<sub>2</sub>, 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 MgCl<sub>2</sub>, 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  $\mu$ L of elution buffer (0.1 M NaHCO<sub>3</sub>,  
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  $\mu$ 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  $\mu$ L TE and 1  $\mu$ 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  $\mu$ 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  $100 * e^{-(Ct_{IN} - \log_2(DF) - Ct_{IP})}$ , with  $e$  the efficiency of the primer pair,  $Ct_{IN}$  the average  $Ct$  value for the Input  
886 sample,  $DF$  the dilution factor and  $Ct_{IP}$  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](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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945

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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
<b>Parent flower</b>	<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%)

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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  $\mu$ 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  $\mu$ 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  $\mu$ 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  $\mu$ 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 Jaspar, 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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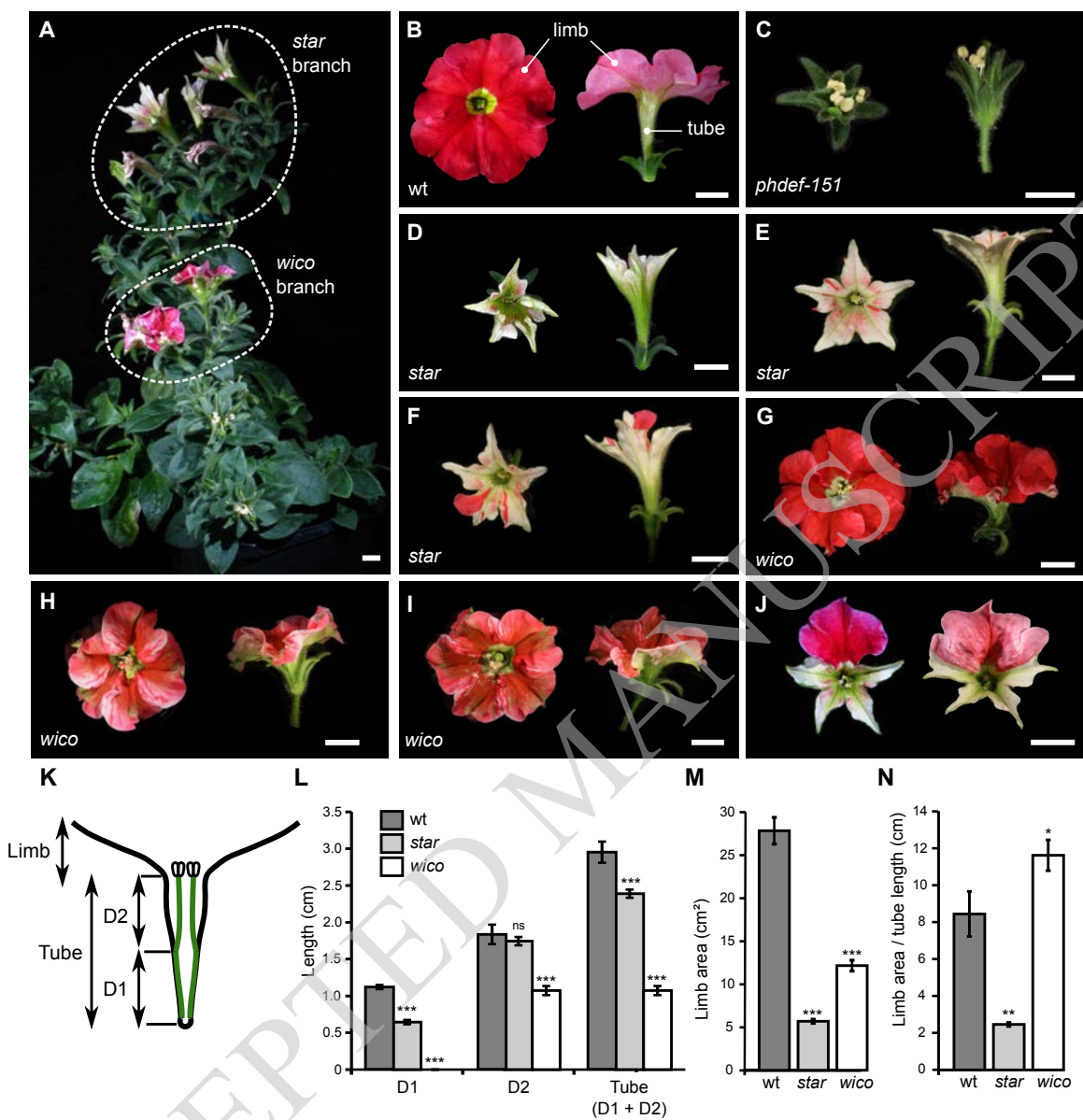
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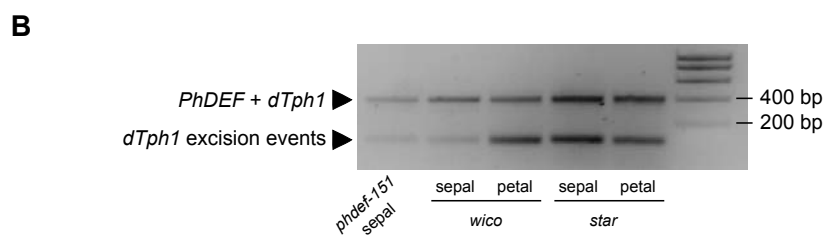
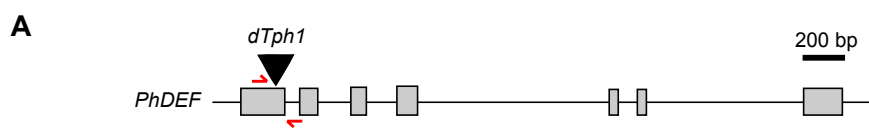


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**Figure 1. Macroscopic description of the *star* and *wico* flowers.**

(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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**C**

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

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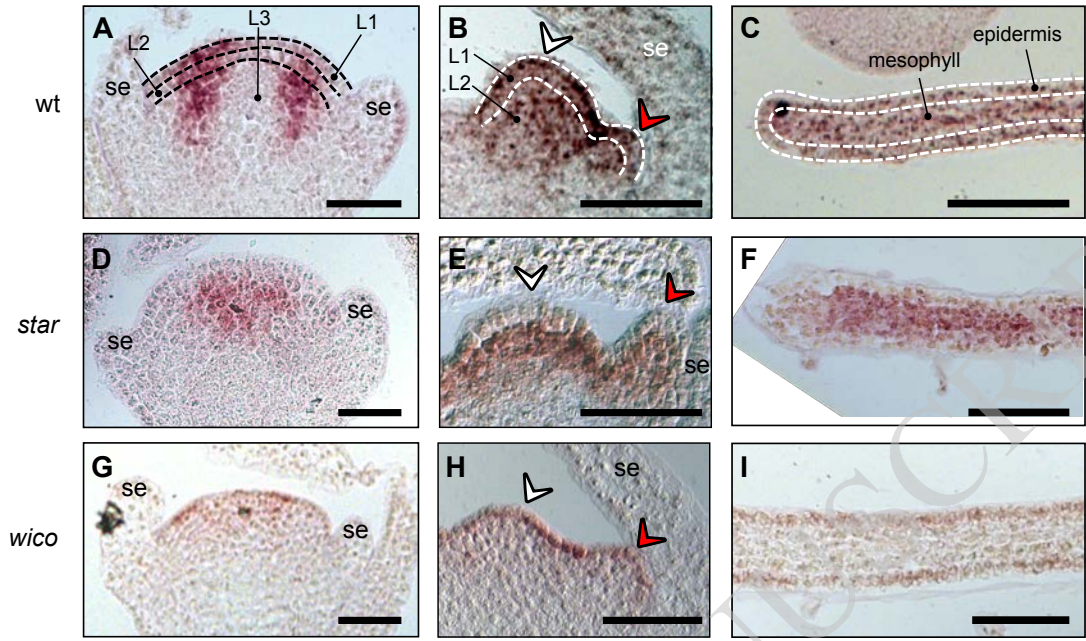
**Figure 2. Sequencing the *PhDEF* excision alleles in *star* and *wico* flowers.**

**(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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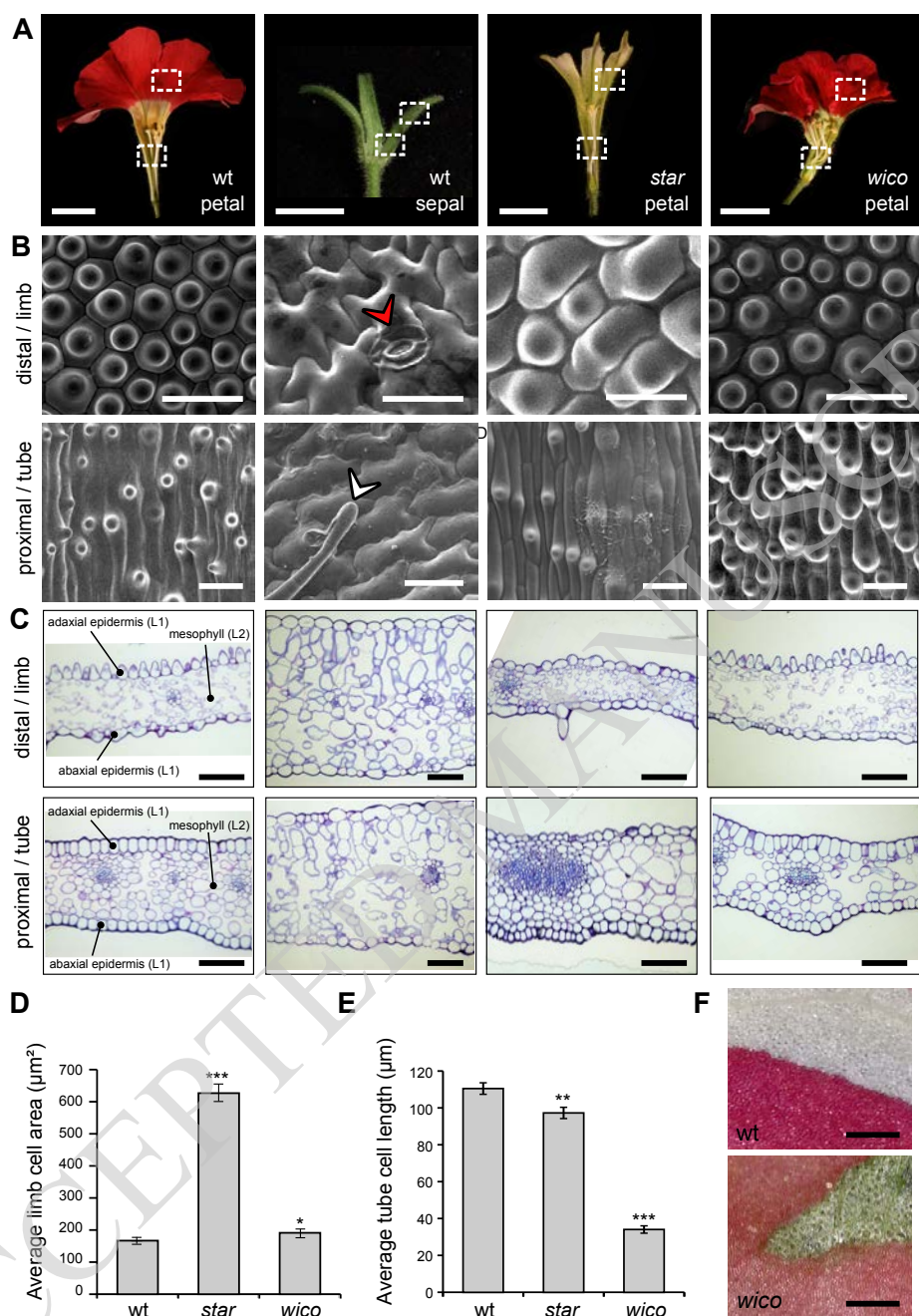


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**Figure 3. Localization of the *PhDEF* transcript in wt, *star* and *wico* flowers by *in situ* hybridization.**

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  $\mu$ m.

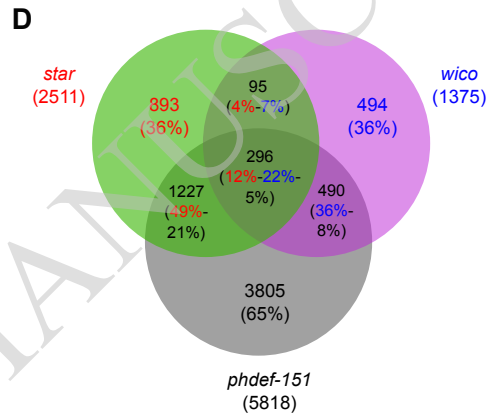
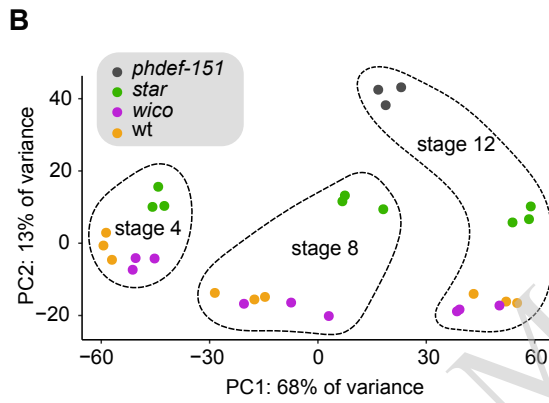
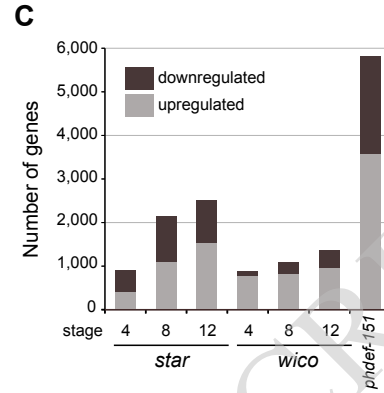
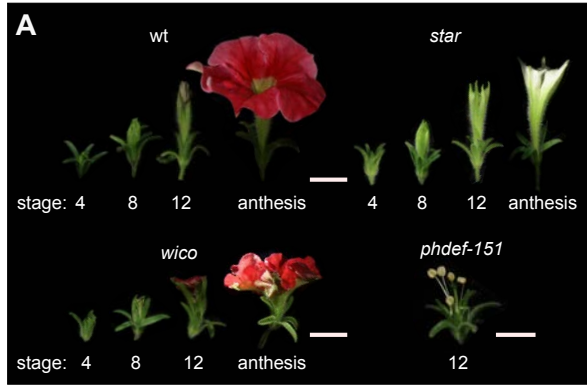
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**Figure 4. Epidermal and mesophyll cell identities in wt petals and sepals, and *star* and *wico* petals.**

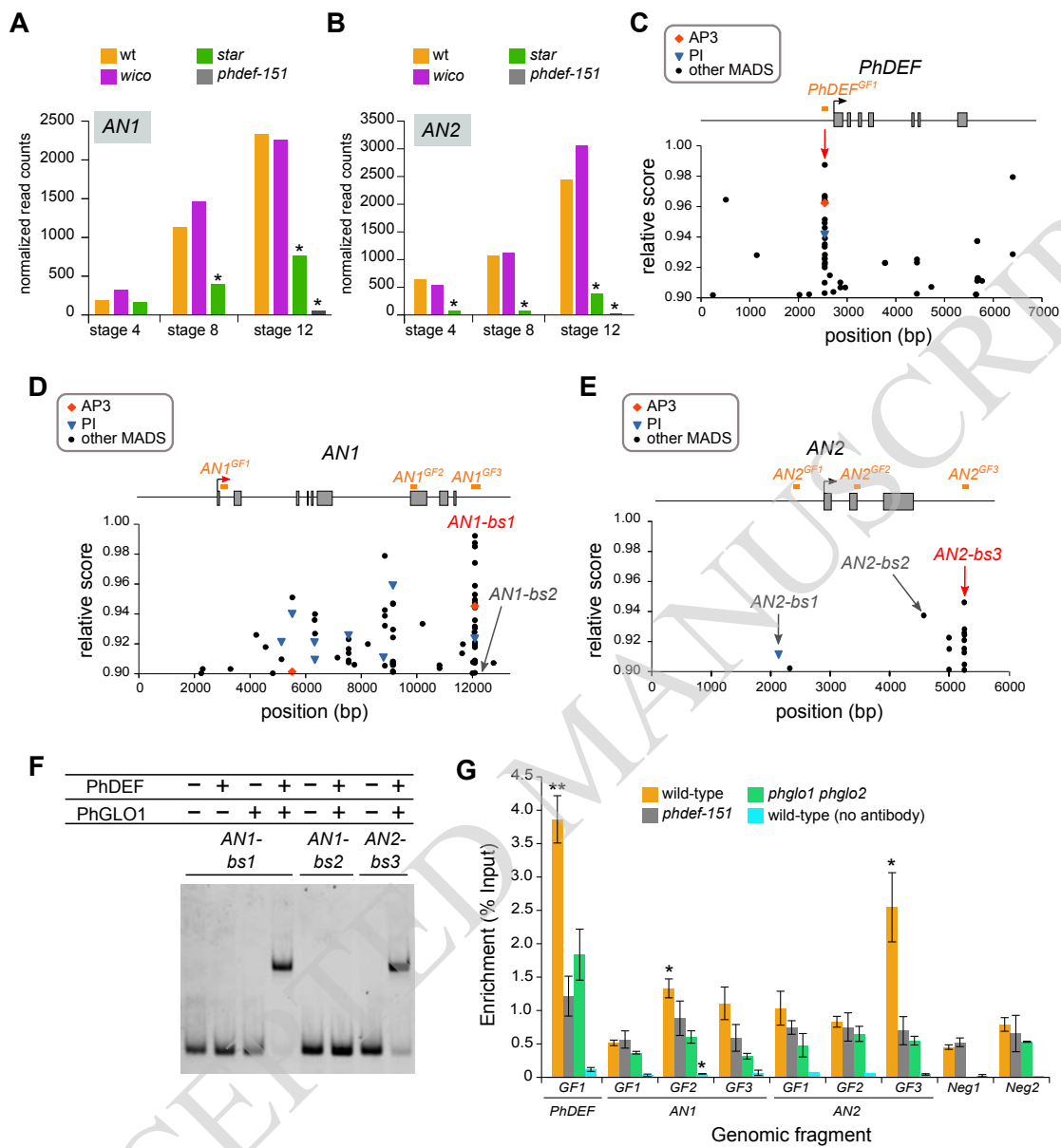
**(A)** From left to right: wild-type (wt) petals, wt sepals, *star* petals and *wico* petals cut open longitudinally to show areas used for scanning electron microscopy and cross-sections. Petals were subdivided into limb and tube area, and sepals were subdivided into a distal and a proximal part, as 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  $\mu\text{m}$ . **(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  $\mu\text{m}$ . **(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  $\mu\text{m}$ .



**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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