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RESEARCH ARTICLE

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

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Short title: Role of cell layers in petal development

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

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ABSTRACT

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

IN A NUTSHELL

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

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

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

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

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

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

In petunia (Petunia x hybrida, abbreviated Ph for gene names), gene duplication has generated four B-class genes, namely PhDEF (DEFICIENS) and PhTM6 (TOMATO MADS-BOX GENE6) belonging to the DEF/AP3 subfamily, and PhGLO1 (GLOBOSA1) and PhGLO2 (GLOBOSA2) belonging to the GLO/PI subfamily (Vandenbussche et al., 2004; Rijpkema et al., 2006; van der Krol et al., 1993; Angenent et al., 1992). Mutating the two members of each subfamily (phdef phtm6 or phglo1 phglo2 double mutants) results in a classical B-function mutant phenotype with homeotic transformation of petals into sepals and stamens into carpels (Vandenbussche et al., 2004; Rijpkema et al., 2006). Additionally, gene copies within the DEF/AP3
subfamily have diverged in function: while *PhDEF* exhibits a classical B-class expression pattern largely restricted to developing petals and stamens, *PhTM6* is atypically expressed in stamens and carpels, and its upregulation depends on the petunia C-function genes (Rijpkema et al., 2006; Heijmans et al., 2012a). As a consequence, the single *phdef* mutant displays a homeotic conversion of petals into sepals, while the stamens are normal due to functional redundancy with *PhTM6* (Rijpkema et al., 2006). The petunia *phdef* mutant is therefore an interesting model to study the mechanism of petal identity specification alone since it displays a single-whorl complete homeotic transformation, which is quite rare for floral homeotic mutants that generally show defects in two adjacent whorls.

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

Although the petunia petal tube and limb seem to play important ecological roles, the mechanisms driving their development are mostly unknown. Tube and limb develop as relatively independent entities in flowers from the Solanaceae family, to which petunia belongs: for instance, tube length and limb width are uncorrelated traits in intra-specific crosses performed in *Nicotiana* or *Jaltomata* (Bissell and Diggle, 2008; Kostyun et al., 2019). Moreover, tube and limb identities can be acquired independently: this is strikingly observed in the petunia *blind* mutant, a partial A-class mutant that forms an almost wild-type tube topped by functional anthers, due to ectopic C-class activity in the second whorl (Cartolano et al., 2007). Apart from the petal identity genes, the molecular players involved in petunia tube or limb morphogenesis are mostly unknown. General growth factors affect petal development as a whole (both tube and limb) together with other vegetative or reproductive traits (Vandenbussche et al., 2009; Terry et al., 2019; Brandoli et al.,
2020), but very few genes have been found to specifically affect growth of one subdomain of the petal (Zenoni et al., 2004). Therefore, the mechanisms of petunia tube and limb morphogenesis remain to be fully explored.

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

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

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

Previously described null alleles for the PhDEF gene (also named GP or pMADS1) were obtained by either ethyl methanesulfonate (EMS) mutagenesis (de Vlaming et al., 1984; Rijpkema et al., 2006) or by γ-radiation (van der Krol et al., 1993). Because neither of these alleles were straightforward to genotype in a heterozygous state, we screened our sequence-indexed dTph1 transposon mutant population in the W138 genetic background (Vandenbussche et al., 2008) for other insertions into PhDEF. We identified a mutant allele named phdef-151, referring to the dTph1 insertion 151 bp downstream of the ATG in the first exon of the PhDEF gene, predicted to fully disrupt the MADS-domain in the protein sequence by premature termination of the first exon due to multiple stop codons in the different reading frames of dTph1. As observed for previously identified phdef null alleles, phdef-151 flowers display a complete homeotic conversion of petals into sepals, while heterozygous or homozygous wild-type siblings display red-coloured wild-type petals (Fig. 1A-C). phdef-151 is thus very likely a null mutant allele.

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

We quantified the changes in flower morphology (Fig. 1K-N) and found that total limb area was reduced almost 5-fold in star flowers (Fig. 1M). In contrast, total tube length was only slightly reduced (by 19%) in star as compared to wt (Fig. 1L), and this was mainly due to a reduction in length of domain D1, corresponding to the part of the tube fused with stamens (as defined in (Stuurman et al., 2004), Fig. 1K), while length of the rest of the tube (domain D2) remained
unchanged (Fig. 1L, Supplemental Figure S2). As a result, the ratio between limb area and tube length, which we use as a simple measure for overall corolla morphology, is reduced about 4-fold in star flowers as compared to wt (Fig. 1N). In addition, we occasionally observed fully pigmented secondary revertant sectors of various sizes in the star genetic background, in some cases leading to the development of a single wt-like petal in a star flower background (Fig. 1J). These revertant sectors, observed multiple times, always exhibited simultaneous restoration of pigmentation and normal petal limb growth patterns, demonstrating that the strongly reduced pigmentation in star petals was due to impaired PhDEF function, and not to an additional mutation in the pigmentation pathway.

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

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

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

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

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

The wico flowers are L1 periclinal chimeras

Excision of dTph1 from a gene can occur at different times during plant development: if happening at the zygotic stage, then the whole plant will have a dTph1-excised allele. If excision occurs later, this will result in a genetic mosaic (chimera) with a subset of cells carrying the dTph1 insertion at the homozygous state and others having a dTph1-excised allele. This typically leads to branches or
flowers with a wt phenotype on a mutant mother plant (assuming a recessive mutation). Furthermore, since all plant organs are organized in clonally-independent cell layers, excision can happen in one cell layer only, thereby creating a periclinal chimera, i.e. a branch or flower where cell layers have different genotypes (Frank and Chitwood, 2016; De Keukeleire et al., 2001).

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

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

The star flowers are L2 periclinal chimeras

Similarly, we analyzed the progeny of the star flowers, and the star phenotype was also not heritable, and hence maintained by cuttings of revertant branches. The progeny of the star flowers with a PhDEF+6 allele yielded three different phenotypic classes (in a proportion close to 1:1:2; Table 1): plants displaying a phdef phenotype, plants having wt flowers, and plants carrying flowers
with a wild-type architecture but with altered pigmentation, further referred to as « pink wt » (Supplemental Figure S4).

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

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

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

Having determined the genetic basis of the star and wico phenotypes, we next wondered how layer-specific PhDEF expression affects the determination of cell identity, in the layer where PhDEF is expressed (cell-autonomous effect) but also in the layer devoid of PhDEF expression (non-cell-autonomous effect). For this, we observed petal adaxial epidermal cells by scanning electron microscopy, and mesophyll cells on petal cross-sections, in wt petals and sepals, and in star and wico petals (Figure 4).
On the adaxial side of the wt petal (Fig. 4A), cells from the limb are round and conical as in many angiosperm petal limbs, while cells from the tube are elongated with a central cone (Fig. 4B) (Cavallini-Speisser et al., 2021). In contrast, the adaxial epidermis of wt sepals (indistinguishable from phdef-151 second whorl organs) displays typical leaf-like features (Morel et al., 2019), with puzzle-shaped cells interspersed with stomata and trichomes (Fig. 4B). Epidermal cell identity can thus be clearly determined on the basis of cell shape. In wico petals, epidermal limb cells are conical, similar to wt cells from the same area, although marginally bigger (Fig. 4B, D). In contrast, cells from the tube, albeit displaying a similar shape to wt cells, are strongly reduced in length (Fig. 4B, E), suggesting that a defect in cell elongation is at least partly responsible for tube length reduction in wico petals.

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

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

In star petals, the mesophyll strongly resembles a wt petal mesophyll in its organization, however cells are bigger and more densely packed in the tube, suggesting that PhDEF activity in the L2 layer is not entirely sufficient to specify normal mesophyll formation in the tube, which might be linked to the slightly reduced size of the tube of star flowers (Fig. 1L). In wico petals, mesophyll cells appeared very similar to wt and their organization was clearly distinct from the one
found in sepals since no palisade layer was observed. However, peeling the epidermis from wico limb revealed that the underlying mesophyll harbored chloroplasts, similar to a sepal mesophyll and in striking contrast with the white mesophyll of wt petal limb (Fig. 4F). Thus, the phdef mutant mesophyll in wico flowers has an intermediate identity between sepal and petal. In summary, our results show that for most features, PhDEF directs petal cell identity autonomously, and that non-autonomous effects also influence cell identity across layers. The interpretation of these effects is summarized in Supplemental Figure S6. In contrast, the observation of star revertant sectors (Supplemental Figure S5) revealed that cell identity is entirely defined autonomously within the epidermal layer, since a sharp transition in cell pigmentation, size and shape is observed in these sectors (Supplemental Figure S5). This suggests that different processes are at stake for cell-cell communication of petal identity across and within layers.

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

**Transcriptome sequencing of star and wico petals**

To better understand the molecular basis for the star and wico phenotypes, we performed RNA-Seq on total petal tissue at three developmental stages, including wt and phdef-151 samples (Figure 5). We chose an early stage (stage 4 as defined in (Reale et al., 2002)), an intermediate stage (stage 8) when tube length is at half its final size, and a late stage (stage 12) before limb is fully expanded (Fig. 5A). For phdef-151 we only sequenced second-whorl sepal tissue at stage 12 (before anthesis). Principal component analysis showed that developmental stage is the first contributor to variation in gene expression, while genotype corresponds to the second axis of variation (Fig. 5B). All genotypes clustered separately except wico and wt samples which were highly similar at the two later stages. We analyzed one-to-one differential gene expression between mutant and wt samples with DESeq2 (Love et al., 2014) and we found on average 5,818 differentially expressed genes (DEGs) in phdef-151, as compared to 1,854 and 1,115 DEGs in star and wico respectively, when averaging for all stages (Fig. 5C, Supplemental Dataset S1).
There were generally more upregulated genes than downregulated ones in mutant or chimeric genotypes, and the number of DEGs increased as development progressed in the petal in both *star* and *wico* (Fig. 5C). At stage 12, a large proportion of DEGs (58-61%) in *wico* or *star* petals were also differentially expressed in *phdef-151* (Fig. 5D), as expected since *wico* and *star* flowers are mutant for *PhDEF* in one cell layer. Genes uniquely differentially expressed in *star* or *wico* flowers represented 36% of DEGs for each, and only 16-29% of DEGs were jointly differentially expressed in *star* and *wico* flowers, consistent with the very different phenotypes of these flowers. These proportions indicate that the *star* and *wico* phenotypes are mostly subtended by the differential expression of sets of genes also differentially expressed in *phdef-151*, together with the differential expression of a unique set of genes for each genotype.

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

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

The *star* and *wico* periclinal chimeras have revealed layer-specific roles of *PhDEF* in the establishment of petal identity and petal development. More specifically, the major layer-specific phenotypes that we have identified are petal pigmentation, conical cell formation and limb growth (controlled by the epidermal-specific expression of *PhDEF*), and tube growth (controlled by the mesophyll-specific expression of *PhDEF*). Therefore, our chimeras show the potential to further explore the exact nature of the link between layer-specific *PhDEF* activity and layer-specific phenotypes. As a proof-of-concept, we explored if *PhDEF* could directly control petal pigmentation
in the limb epidermis. Pigmentation appeared to us as a trait of choice, since its regulatory and biosynthetic factors are well described, while this was not the case for the other traits mentioned above. Moreover, the absence of pigmentation in star petals, the restoration of pigmentation in L1-revertant sectors and the phenotype of the pink wt flowers all converged to a direct link between PhDEF expression in the epidermis and petal pigmentation.

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

Indeed, the an1 mutant has fully white petals and the an2 mutant has strongly reduced limb pigmentation (Quattrocchio et al., 1999; Spelt et al., 2000). Furthermore, AN2 was shown to act as an upstream activator of AN1 since overexpressing AN2 in petunia leaves is sufficient to activate AN1 expression, and for anthocyanins to accumulate (Quattrocchio et al., 1998; Spelt et al., 2000). We observed that both genes were already expressed at stage 4 of wt petal development, before any pigmentation is visible, and their expression levels strongly increased from stage 4 to stage 12, while both being strongly downregulated in star petals and phdef-151 second whorl organs, but not in wico flowers (Fig. 6A, B). AN2 was expressed at higher levels than AN1 at all stages, consistent with its most upstream role in the anthocyanin pigmentation pathway.

We aimed to test if PhDEF could directly bind to AN1 and AN2 genomic sequence, potentially to regulate their expression. For this, we first attempted to predict PhDEF binding on the genomic sequences of AN1 and AN2. We used the high-quality transcription factor (TF) binding profile database Jaspar (Fornes et al., 2020; Sandelin et al., 2004), using position weight matrices for each TF to compute relative binding scores that reflect in vitro binding preferences (Stormo, 2013). The exact DNA-binding specificity of PhDEF has not been characterized, but that of its
Arabidopsis homologs AP3 and PI has been (Riechmann et al., 1996b). However, since PhDEF DNA-binding specificity might be slightly different to those of AP3 and PI, we decided to predict binding for all MADS-box TFs available in Jaspar 2020, accounting for 23 binding profiles including those of AP3 and PI (Fornes et al., 2020). We hypothesized that sequences predicted to be bound by several MADS-box TFs were putative CArG boxes (the binding site for MADS-box proteins, whose canonical sequence is CC(A/T)nGG, but real binding sites show some variation to this consensus (Aerts et al., 2018)).

As a validation of this strategy, we analyzed the genomic sequence of PhDEF and found a putative CArG box in the PhDEF promoter (visible by the presence of good predicted binding sites for several MADS-box proteins and therefore appearing as a clear black line in Fig. 6C). This CArG box has been validated in the literature: it is highly conserved between distantly-related flowering plants (Rijpkema et al., 2006) and it was shown to be important for AP3 petal-specific expression and for its auto-activation in Arabidopsis (Hill et al., 1998; Wuest et al., 2012), and for DEF function and binding to its own promoter in Antirrhinum (Schwarz-Sommer et al., 1992). We next applied this predictive approach to the genomic sequences of AN1 and AN2. For AN1, we predicted a putative CarG box (ANI-bs1) with a very high score for several MADS-box proteins and for AP3 and PI in particular, in the terminator region (Fig. 6D). For AN2, we also predicted one putative CArG box (AN2-bs3), again in the terminator region of the gene (Fig. 6E), although its binding score was more modest in comparison to AN1-bs1. The sequence of AN1-bs1 corresponds to a close-to-canonical CArG box (CTATATTTGG) and the sequence of AN2-bs3 corresponds to a perfectly symmetrical canonical CArG box (CCATAATAGG).

To determine if PhDEF could indeed bind to ANI-bs1 and AN2-bs3 and potentially regulate AN1 and AN2 expression, we performed gel shift assays using in vitro translated PhDEF and/or PhGLO1 proteins (Fig. 6F). We found that, when incubating a 60-bp fragment containing AN1-bs1 in its center with either PhDEF or PhGLO1, no shift in migration was visible, indicating that neither protein could bind to this site alone. However, when incubating AN1-bs1 with both PhDEF and PhGLO1 proteins, we observed a clear shift in migration, consistent with the obligate heterodimerization of these proteins necessary for DNA binding (Riechmann et al., 1996a). Similarly, a 60-bp fragment containing AN2-bs3 in its center, incubated with PhDEF and PhGLO1 proteins, resulted in a clear shift in migration. In contrast, a control 60-bp fragment named AN1-bs2, located in the AN1 terminator region but predicted to have a very low binding score (relative score under 0.8 both for AP3 and PI), was not bound by the PhDEF + PhGLO1 protein complex, showing
that our assay was specific. Therefore PhDEF, when dimerized with PhGLO1, is able to bind to sites in putative regulatory regions in AN1 and AN2, suggesting that it might directly regulate the expression of these two genes.

Next, we tested if PhDEF could bind in vivo to genomic regions containing AN1-bs1 and AN2-bs3 by chromatin immunoprecipitation (ChIP). We produced recombinant PhDEF protein devoid of its highly conserved MADS domain, to avoid cross-reactivity with other MADS-box proteins, and generated a polyclonal antibody against this truncated PhDEF protein. We performed the ChIP assay on second whorl organs (petal or sepal) from wt, phdef-151 or phglo1 phglo2 plants at an intermediate stage of development (stage 8). In wt petal samples, we found a significant binding enrichment for some of the genomic fragments (GF) that we tested, and in particular PhDEFGF1 (Fig. 6G), containing the validated CArG box previously described (Fig. 6C), which is expected since PhDEF activates its own expression. We also observed a significant binding enrichment in AN2GF3 (Fig. 6G), containing the previously identified AN2-bs3 binding site (Fig. 6E). In contrast, no strong enrichment was detected in the ANI genomic fragment containing the ANI-bs1 strong in vitro binding site for PhDEF (AN1GF3). Our ChIP assay was specific, since no enrichment was detected for the phdef-151 mutant, nor for the phglo1 phglo2 mutant (Fig. 6G). The phglo1 phglo2 samples constitute an indirect control for PhDEF binding, since the PhDEF protein partners PhGLO1/PhGLO2 are absent, thereby indirectly preventing PhDEF binding on DNA. The fact that we do not detect any binding enrichment in these plants shows that our ChIP assay is robust. Therefore, we conclude that PhDEF binds to the terminator region of AN2 in planta, and that PhDEF is a putative direct regulator of AN2 expression in the petal epidermis.
DISCUSSION

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

Contribution of cell layers to mature petunia petals

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

In fact, the contribution of cell layers to mature organ organization can only be strictly assessed by clonal analysis, where one follows cell lineage using trackable cell-autonomous markers. In petunia, no clonal analysis has been performed so far, hence one can only assume which cell layers participate in petal development based on clonal analyses performed in closely-related species. In Datura, periclinal chimeras induced by colchicine treatment and refined histological observations have provided a detailed clonal analysis for cell layers in floral organs (Satina and Blakeslee, 1941). The first visible event of petal initiation is a periclinal cell division from the L2 layer, and further growth of the petal depends primarily on cell divisions from the L2, both anticlinal and periclinal. The L3 layer only contributes to the vascular tissue at the very base of the petal. L1-derived cells form the epidermis by anticlinal divisions, except at the petal edges where periclinal divisions are observed, leading to L1-derived cells invading the mesophyll. Hence, the
Datura petal is formed by all 3 layers with a major contribution of the L1 and L2 layers, and a relative enrichment in L1-derived cells (by thinning of the mesophyll) progressing from the base towards the tip of the petal. In this work, we hypothesized that the petunia petal is formed similarly. Accordingly, we only obtained two phenotypic classes of periclinal chimeras, star and wico, suggesting that L3-specific PhDEF expression probably only leads to a phdef mutant phenotype.

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

Different cell layers drive tube and limb morphogenesis

The star and wico phenotypes revealed that in petunia petals, the epidermis is the main driver for limb morphogenesis while the mesophyll is the main driver for tube morphogenesis. The epidermis has been proposed to be the layer in control of organ morphogenesis, since it is a layer under tension that restricts growth of the underlying inner tissues that tend to expand (Kutschera and Niklas, 2007). In particular, epidermal expression of the brassinosteroid receptor BRI1 (BRASSINOSTEROID INSENSITIVE 1) is sufficient to restore normal leaf morphogenesis in a bri1 mutant (Savaldi-Goldstein et al., 2007). Similarly, the expression of the auxin transporter PIN1 (PIN-FORMED 1) in the L1 of the SAM is sufficient to restore normal phyllotaxis in a pin1 mutant (Kierzkowski et al., 2013). However, pieces of evidence suggest that organ inner layers can have an active role in morphogenesis: for instance, mesophyll-specific expression of ANGUSTIFOLIA (AN) is sufficient to restore normal leaf width in the Arabidopsis an mutant (Bai et al., 2010); leaf shape is controlled by the L2- and L3-derived tissues in Nicotiana glauca (McHale and Marcotrigiano, 1998); and the leaf mesophyll is the main player for leaf flatness in Arabidopsis (Zhao et al., 2020).
Moreover, expressing BRI1 in the root phloem also restores bri1 plant dwarfism (Graeff et al., 2020). The contribution of cell layers to organ morphogenesis is thus a complex process that varies between organs, species and the genetic systems investigated.

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

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

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

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

Alternatively, the non-autonomous effects that we observed might be triggered by mechanical signals transmitted between layers. For instance, in star flowers normal growth of the mesophyll could merely drag along epidermal cells, since cells are connected by their cell walls, which could be sufficient to trigger their expansion and division. Other features, like conical cell shape, might be directly influenced by mechanical signals. Indeed, conical cells are shaped by a circumferential microtubule arrangement controlled by the microtubule-severing protein KATANIN, and altering this arrangement affects conical cell shape (Ren et al., 2017). Microtubule arrangement responds to mechanical signals (Hamant et al., 2008), which are likely to be
transmitted between layers. Therefore, it is possible that the formation of bulging cells in the *star*
epidermis is merely triggered by mechanical signals from the growing underlying layer, independent of any petal identity specifier, as was recently evidenced from the observation of conical-like bulges on the hypocotyl of the tubulin kinase mutant *nek6* (Takatani et al., 2020). The molecular or physical nature of the signals involved in communication between layers remains to be explored in full depth.

Towards the gene regulatory networks of petal development

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

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

Finally, we investigated the direct link between PhDEF and petal pigmentation and found that, *in vitro*, the PhDEF + PhGLO1 protein complex directly binds to predicted binding sites in the regulatory regions of *AN1* and *AN2*. We confirmed that PhDEF binds to the corresponding genomic region of *AN2 in planta* by ChIP, but not for *AN1*, confirming that *in vitro* binding does not necessarily imply *in vivo* binding, the last being strongly influenced by the local chromatin landscape. The binding site of PhDEF that we identified on *AN2* (*AN2-bs3*) lies in the terminator
region of the gene (and the next gene on the chromosome is more than 100 kb away), which was surprising since around 80% of MADS binding sites are located within the 3 kb promoter region of their target genes (Aerts et al., 2018). However, the presence of a binding site in the terminator region is still compatible with an activating role in transcription, through DNA looping to the promoter (Jash et al., 2012) or by promoting transcription termination and reinitiation (Wang et al., 2000). Other putative CArG boxes in the genomic region of AN2 are AN2-bs1, located 866 bp upstream the ATG in the promoter region, and AN2-bs2, located 62 bp downstream the STOP codon in the 3’UTR region. Both have non-canonical CArG box sequences (GAAAAGTAG for AN2-bs1 and TCTTTTTTAA for AN2-bs2) and were not bound in our gel shift assay (Supplemental Figure S8). Still, it is possible that regulators other than MADS-box TFs could form protein complexes with PhDEF and mediate looping to the promoter region of AN2. The precise mechanism by which PhDEF might activate AN2 transcription remains to be uncovered.

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

The fact that we detected strong in planta binding of PhDEF to AN2, together with the fact that AN2 expression is strongly down-regulated in the phdef-151 transcriptome, suggests that PhDEF is a good candidate to directly activate AN2 expression in the petal. Ectopic expression of AN2 in petunia leaves is sufficient to trigger anthocyanin accumulation in this tissue, by inducing AN1 expression among others (Spelt et al., 2000; Quattrocchio et al., 1998). Therefore, if PhDEF indeed activates AN2 expression, it should be sufficient to launch the whole pigmentation pathway in the wt petal limb. However, to fully support this conclusion, functional tests on the role of PhDEF binding to AN2-bs3 in regulating AN2 expression should be conducted. A direct link between petal identity and pigmentation has yet to be established, although genetic evidence in orchid flowers strongly implied that different B-class proteins heteromeric complexes are responsible for specific pigmentation spots in the different petal types, but physical binding of these
B-class protein complexes on pigmentation genes was not tested (Hsu et al., 2021). The direct target genes of B-class proteins have been identified by ChIP-Seq and transcriptomic analyses in Arabidopsis (Wuest et al., 2012), but this species has unpigmented petals, thereby preventing us to draw any possible link between petal identity and pigmentation. The petunia petal is the ideal system to test this direct link, and our results suggest that PhDEF might be the direct link between petal identity and its epidermal pigmentation.
MATERIALS AND METHODS

Plant materials, growth conditions and plant phenotyping

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

Genotyping

Extraction of genomic DNA from young leaf tissue was performed according to (Edwards et al., 1991). The region spanning the dTpH1 insertion site in PhDEF was amplified using primers MLY0935/MLY0936 (Supplemental Table S2). PCR products were separated on a 2% (w/v) agarose gel, fragments of interest were purified using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel), and sequenced with Eurofins SupremeRun reactions.

In situ RNA hybridization

Floral buds from wt, 2 wico and 1 star lines were fixed overnight in FAA (3.7% (v/v) formaldehyde, 5% (v/v) acetic acid, 50% (v/v) ethanol), cleared in Histo-clear and embedded in paraffin to perform 8 μm sections. PhDEF cDNA sequence was amplified from wt petunia inflorescence cDNAs with primers MLY1738/MLY1739 (Supplementary Table 2), generating a 507 bp fragment excluding the part encoding the highly conserved DNA-binding domain. The digoxigenin-labeled RNA probe was synthesized from the PCR fragment by in vitro transcription, using T7 RNA polymerase (Boehringer Mannheim). RNA transcripts were hydrolyzed partially for 42 min by incubation at 60°C in 0.1 M Na$_2$CO$_3$/NaHCO$_3$ buffer, pH 10.2. Later steps were performed as
described by (Cañas et al., 1994). For imaging, slides were mounted in Entellan (Sigma) and
imaged with a Zeiss Axio Imager M2 light microscope equipped with a Zeiss Axio Cam HRc
camera.

**Petal cross-sections**

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

**Scanning electron microscopy (SEM)**

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

**RNA-Seq**

Petal tissue was collected at 1 pm from several plants stemming from a single *star* line, a single
*wico* line, and several individual wt plants (progeny of a single *star* flower) and *phdef-151* plants
(progeny of the same *star* flower). Tube length was macroscopically measured to compare stages,
the corolla was cut open and stamens were removed as much as possible from the corolla by pulling
on the filaments fused to the tube. One biological replicate contains total petal tissue from 2
flowers. Tissue was ground in liquid nitrogen and RNA was extracted with the Spectrum Plant Total
RNA Kit (Sigma) including on-column DNase digestion (Sigma). RNA integrity and quantity were
determined by a Bioanalyzer RNA 6000 Nano assay (Agilent). Libraries were prepared with poly-A enrichment and single-end 75-bp sequencing was performed on a NextSeq 500 platform (Illumina). 16 to 23 million reads were recovered per library. Reads were checked for quality with FastQC v0.11.4 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), adaptors and low-quality ends were trimmed with Cutadapt v 1.16 (Martin, 2011) and custom Perl scripts. The reference genome sequence used for transcriptome analysis is the *Petunia axillaris* v1.6.2 HiC genome published in (Bombarely et al., 2016) and further scaffolded by HiC by DNAzoo (Dudchenko et al., 2017, 2018); gene annotations were transferred from the published assembly to the HiC-scaffolded version using Blat (Kent, 2002), Exonerate (Slater and Birney, 2005) and custom Perl scripts. In the rare cases when gene annotations from the published genome mapped to several regions in the HiC-scaffolded genome, these different putative genes were identified by a letter added at the end of the gene identifier (for instance Peaxi162Scf00179g00121a). The complete set of reads was mapped on the reference genome sequence using HISAT2 v2.2.1 (Kim et al., 2015) to identify splicing sites, before performing mapping sample per sample. Reads per gene were counted using FeatureCounts v1.5.1 (Liao et al., 2014). DESeq2 version 3.12 (Love et al., 2014) was used with R version 4.0.3 to perform the Principal Component Analysis and the differential gene expression analysis. Genes having less than 10 reads in the sum of all samples were considered as non-expressed and discarded. Genes were considered to be differentially expressed if log2FoldChange > 1 or < -1, and p-adjusted value < 0.01. The bioinformatic pipeline for annotation transfer, read cleaning, splicing site discovery, read mapping and preliminary DESeq2 results can be found at gitbio.ens-lyon.fr/rdp/petunia_star_wico_rnaseq. Venn diagrams were built with InteractiVenn (Heberle et al., 2015). Due to the automatic gene name annotation pipeline used in (Bombarely et al., 2016) based on homology with tomato (*Solanum lycopersicum*) proteins, many of the previously characterized petunia genes have not been annotated according to their first described name, making interpretation of some of the RNA-Seq results less straightforward. We have manually added annotations of 42 genes from the anthocyanin biosynthesis pathway based on the Supplementary Note 7 from (Bombarely et al., 2016), and 31 type-II MIKC-C MADS-box genes based on previous studies from the literature; these annotations can be found in the Supplemental Dataset S1 of this manuscript. We noticed that the gene annotations from three major pigmentation genes, *DFR* (*DIHYDROFLAVONOL-4-REDUCTASE*, Peaxi162Scf00366g00630), *CHSa* (*CHALCONE SYNTHASE a*, Peaxi162Scf00047g01225) and *PHI* (Peaxi162Scf00569g00024) were lost during the gene annotation transfer procedure, because they lie in regions of the genome that are still
poorly resolved. Therefore, we manually searched the position of these transcripts in the HISAT2 output and we were able to map part of the DFR and CHSa genes to two small scaffolds, while PHI position was not found. We added the transcript positions of DFR and CHSa in the gtf/gff files before running FeatureCounts. The read counts for DFR and CHSa reported in Supplemental Figure S7 are therefore an under-estimation of their actual expression levels, since we miss part of the genes.

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

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

**Electrophoretic mobility shift assays (EMSAs)**

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

**PhDEF protein and antibody production**

The PhDEF truncated cDNA (without the sequence coding for the MADS domain) was chemically-synthesized with optimization for expression in Escherichia coli and cloned into a pT7 expression vector by Proteogenix (www.proteogenix.science). The expected PhDEF protein starts at amino acid 60 (PSITT...) and ends at the last amino acid of the sequence (...FALLE), and a 6xHis tag was added at the N-terminal part of the protein. The 6xHis-PhDEF protein was purified by affinity
column with a Nickel resin under denaturing conditions (8M urea) by Proteogenix. The purified protein was injected in two rabbits for immunization by Proteogenix, to generate PhDEF-directed polyclonal antibodies, that were purified by affinity against the antigen. Both lots of purified antibodies were validated by immunoblot in petal or sepal tissues from wt, *phdef-151* and *phtm6* samples.

**Chromatin immunoprecipitation (ChIP)**

One biological replicate comprises the full corolla from 2 flowers (wt), second whorl sepals from 3 flowers (*phdef-151*) or second whorl sepals from 3 to 4 flowers (*phglo1 phglo2*), and the full experiment was performed for 3 biological replicates for wt and *phdef-151* and 2 biological replicates for *phglo1 phglo2*. Samples at stage 8 were collected and ground in liquid nitrogen. Ground tissue was resuspended into 10 mL fixation buffer (10 mM Hepes pH7.6, 0.5 M sucrose, 5 mM KCl, 5 mM MgCl₂, 5 mM EDTA pH8, Complete Protease Inhibitor Cocktail (Merck), 14 mM 2-mercaptoethanol) and a double cross-linking was performed at room temperature (1 hour with disuccinimidyl glutarate at 2.5 mM with gentle shaking, and 5 minutes with formaldehyde 1% (v/v)). Cross-linking was stopped by adding glycerine at 200 mM and samples were put directly on ice. Cells were lysed with a 40 mL-Dounce tissue grinder (Duran Wheaton Kimble), Triton X-100 was added at 0.6% (w/v) and the lysate was filtered subsequently through 100 µm and 40 µm nylon meshes to recover nuclei. Nuclei were pelleted for 10 minutes at 3,000 g at 4°C, and the pellet was resuspended in 300 µL of cold nuclear isolation buffer (i.e. fixation buffer without 2-mercaptoethanol), carefully deposited on 600 µL of a 15% Percoll solution (15 % (v/v) Percoll, 10 mM Hepes pH8, 0.5 M sucrose, 5 mM KCl, 5 mM MgCl₂, 5 mM EDTA pH8) and centrifuged for 5 minutes at 2,000 g at 4°C. The pellet was resuspended into 900 µL of cold nuclear lysis buffer (50 mM Tris-HCl pH7.5, 0.1% (w/v) SDS, 10 mM EDTA pH8) to lyse the nuclei, and chromatin was sonicated twice for 15 minutes with a Covaris S220 sonicator (peak power 105, Duty factor 5, Cycles/Burst 200 for 900s). For each sample, 25 µL of magnetic protein-A Dynabeads and 25 µL of magnetic protein-G Dynabeads (Invitrogen) were washed twice with 100 µL of cold ChIP dilution buffer (15 mM Tris-HCl pH7.5, 150 mM NaCl, 1% (w/v) Triton X-100, 1 mM EDTA pH8) using a magnetic rack (MagRack 6, Cytiva). Beads were mixed with 2.5 µg of anti-PhDEF antibody and 1.8 mL of cold ChIP dilution buffer, and incubated for 2 hours at 4°C on a rotating wheel. Sonicated chromatin was centrifuged for 5 minutes at 15,000 g at 15°C, and 25 µL of supernatant (for wt samples) or 50 µL of supernatant (for *phdef-151* and *phglo1 phglo2* samples) was added to the mix of beads and antibody, and incubated overnight at 4°C on a rotating wheel. Beads were washed
twice (one quick wash and one long wash with 15 minutes incubation on a rotating wheel) with each of the following buffers: low salt wash buffer (0.1% (w/v) SDS, 1% (w/v) Triton X-100, 2 mM EDTA pH8, 20 mM Tris-HCl pH8, 150 mM NaCl), high salt wash buffer (0.1% (w/v) SDS, 1% (w/v) Triton X-100, 2 mM EDTA pH8, 20 mM Tris-HCl pH8, 500 mM NaCl), LiCl wash buffer (0.25 M LiCl, 1% (v/v) NP40/Igepal, 1% (w/v) deoxycholate, 1 mM EDTA pH8, 20 mM Tris-HCl pH8) and TE buffer. Elution was performed twice with 250 µL of elution buffer (0.1 M NaHCO3, 1% (w/v) SDS) at 65°C. IP and input samples were decrosslinked overnight at 65°C by adding NaCl at 200 mM, then incubating for 2 h at 42°C with 20 µg proteinase K in 10 mM EDTA pH8 and 40 mM Tris-HCl pH6.5. DNA was purified with phenol:chloroform:isoamyl alcohol (25:24:1) followed by chloroform:isoamyl alcohol (24:1), precipitated with ethanol at -20°C and the pellet was washed with ethanol 70%. The dry pellet was recovered in 50 µL TE and 1 µL was used for each qPCR reaction, which was performed in technical triplicates for each biological replicate (3 for wt and phdef-151, 2 for phglo1 phglo2 and the control without antibody). The qPCR reaction was performed with 1X FastStart Universal SYBR Green (Merck) and 0.3 µM primer mix (Supplemental Table S2), for 40 cycles (15 seconds at 95°C, 1 minute at 60°C) in a QuantStudio 6 Flex instrument (ThermoFisher). Percentage of input (enrichment) was calculated as 100* e^(CtIN – log2(DF) – CtIP), with e the efficiency of the primer pair, CtIN the average Ct value for the Input sample, DF the dilution factor and CtIP the average Ct value for the IP sample), as described in (Solomon et al., 2021). The significance of the enrichment was evaluated with a one-tailed t-test comparing the enrichment of the test region to the average of the enrichments of the two negative regions.

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

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

ACCESSION NUMBERS

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

SUPPLEMENTAL DATA

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

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

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

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

Supplemental Figure S5. Epidermal revertant sectors on star petals.

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

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

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

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

Supplemental Table S2. List of primers used in this study.
**Supplemental Dataset S1.** Differential gene expression calculated by DESeq2.

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

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

**FUNDING INFORMATION**

This work was supported by a PhD fellowship to M.C. from the French Ministry of Higher Education and Research, by a grant to Q.C.S. and M.M. from the Agence Nationale de la Recherche (grant ANR-19-CE13-0019, FLOWER LAYER), by a grant to M.M. from IDEXLYON (Université de Lyon, grant ELAN-ERC), and by a grant to V.H. and C.Z. from the Agence Nationale de la Recherche (grant ANR-16-CE92-0023, FLOPINET).

**ACKNOWLEDGMENTS**

We thank Patrice Bolland, Justin Berger and Alexis Lacroix for plant care assistance, the PLATIM platform (SFR BioSciences Lyon, UAR3444/CNRS, US8/Inserm, ENS de Lyon, UCBL) for electron microscopy technical support, Benjamin Gillet and Sandrine Hugues from the sequencing platform of the Institut de Génomique Fonctionnelle de Lyon for library preparation and sequencing of the transcriptomes of this study, Rémy Belois for assistance for in situ hybridization experiments and Daniel Bouyer and Nicolas Dalle for assistance for chromatin immunoprecipitation experiments. We gratefully acknowledge support from the PSMN (Pôle Scientifique de Modélisation Numérique) of the ENS de Lyon for the computing resources.

**AUTHOR CONTRIBUTIONS**

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. performed the experiments. M.C., Q.C.S., J.J., M.V. and M.M. analyzed the data. M.C., C.Z., M.V. and M.M. wrote the article.
Table 1. Progeny of the star and wico flowers after selfing.

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

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<tr>
<td>wico-1</td>
<td>15 (94%)</td>
</tr>
<tr>
<td>wico-2</td>
<td>14 (88%)</td>
</tr>
<tr>
<td>wico-3</td>
<td>16 (100%)</td>
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<tr>
<td>wico-4</td>
<td>15 (94%)</td>
</tr>
<tr>
<td>wico-5</td>
<td>16 (100%)</td>
</tr>
<tr>
<td>wico-6</td>
<td>12 (100%)</td>
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<td>wico-7</td>
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<td>7 (29%)</td>
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<td>star-4</td>
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FIGURE LEGENDS

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 ± s.e.m.

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.
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 µm.

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

(A) From left to right: wild-type (wt) petals, wt sepals, star petals and wico petals cut open longitudinally to show areas used for scanning electron microscopy and cross-sections. Petals were subdivided into limb and tube area, and sepals were subdivided into a distal and a proximal part, as shown by the dotted white rectangles. Scale bar: 1 cm. (B) Representative scanning electron micrographs from the adaxial side of a wt petal, wt sepal, star petal and wico petal (from left to right). The red arrowhead points to a stomata and the white arrowhead points to a trichome. Scale bar: 30 µm. (C) Representative cross-sections from wt petals, wt sepals, star petals and wico petals (from left to right) stained with toluidine blue. The adaxial and abaxial epidermis and the mesophyll are indicated on the wt petal sections. Scale bar: 100 µm. (D) Average limb cell area from the adaxial side of wt, star and wico petals (n = 30 cells). Student's t test with Welch correction, two-sided (* p < 0.05, ** p < 0.01, *** p < 0.005). Error bars represent ± 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 ± s.e.m. (F) Limb area from wt (top) and wico (bottom) petals, after their adaxial epidermis was manually peeled. For wt, the upper half of the picture shows the white underlying mesophyll. For wico, the green triangular area shows the green (chloroplastic) underlying mesophyll. Scale bar: 300 µm.
**Figure 5. Gene differential expression in star and wico petals.**

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

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

(A, B) Expression (as normalized read counts calculated by DESeq2) of AN1 (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 (log2FC < -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), AN1 (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 (AN1-bs1, AN2-bs1, AN2-bs2 and AN2-bs3) and a negative control with a low predicted binding score (AN1-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 AN1-bs1, AN1-bs2 or AN2-bs3 DNA fragments, whose position is depicted in (C-E). Similar results were obtained in 5 additional independent assays for AN1-bs1, 2 additional independent assays for AN2-bs3 and 4 additional independent assays for AN1-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, AN1 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 ± s.e.m.
REFERENCES


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 ± s.e.m.
### Table C

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<td>phdef (+6c)</td>
<td>CCAGTA---------CTGGCAAGCTTCAT</td>
<td>DAKVSIIMISS-TGSGKLHEFIS</td>
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<td>CCAGTACAGCTCTCTGG--------CAAGCTTCAT</td>
<td>DAKVSIIMISSSQSGL--KLHEFIS</td>
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### Diagram A

- dTph1
- PhDEF
- PhDEF + dTph1
- dTph1 excision events

### Diagram B

- PhDEF + dTph1
- dTph1 excision events
- 200 bp
- 400 bp
- sepal
- petal
- wico
- star
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.
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 µm.
Figure 4. Epidermal and mesophyll cell identities in wt petals and sepals, and star and wico petals.

(A) From left to right: wild-type (wt) petals, wt sepals, star petals and wico petals cut open longitudinally to show areas used for scanning electron microscopy and cross-sections. Petals were subdivided into limb and tube area, and sepals were subdivided into a distal and a proximal part, as shown by the dotted white rectangles. Scale bar: 1 cm. (B) Representative scanning electron micrographs from the adaxial side of a wt petal, wt sepal, star petal and wico petal (from left to right). The red arrowhead points to a stomata and the white arrowhead points to a trichome. Scale bar: 30 µm. (C) Representative cross-sections from wt petals, wt sepals, star petals and wico petals (from left to right) stained with toluidine blue. The adaxial and abaxial epidermis and the mesophyll are indicated on the wt petal sections. Scale bar: 100 µm. (D) Average limb cell area from the adaxial side of wt, star and wico petals (n = 30 cells). Student's t test with Welch correction, two-sided (* p < 0.05, ** p < 0.01, *** p < 0.005). Error bars represent ± 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 ± s.e.m. (F) Limb area from wt (top) and wico (bottom) petals, after their adaxial epidermis was manually peeled. For wt, the upper half of the picture shows the white underlying mesophyll. For wico, the green triangular area shows the green (chloroplastic) underlying mesophyll. Scale bar: 300 µm.
Figure 5. Gene differential expression in star and wico petals.

(A) Flowers from wild-type (wt), star, wico and phdef-151 at stages 4, 8 and 12 (only stage 12 for phdef-151), whose petals or sepals were harvested for transcriptome sequencing. Flowers at anthesis are shown for comparison. Scale bar: 1 cm. (B) Principal Component Analysis plot of the samples after analysis of variance with DESeq2, showing that the first principal component corresponds to the developmental stage and the second principal component corresponds to the genotype. (C) Number of upregulated and downregulated genes in star, wico and phdef-151, as compared to wt at the corresponding stages. (D) Venn diagram recapitulating the number of differentially expressed genes (DEGs) in star, wico and phdef-151 petal samples at stage 12, as compared to wt, and their different intersections. Each sector contains the number of DEGs, and between parenthesis is the percentage of genes that it represents from the total number of DEGs in the corresponding sample, with a colour code (red = percentage of DEGs from star samples / blue = from wico samples / black = from phdef-151 samples).
Figure 6. PhDEF binds to AN2 regulatory region in vitro and in vivo.

(A, B) Expression (as normalized read counts calculated by DESeq2) of AN1 (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 (log2FC < -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), AN1 (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 (AN1-bs1, AN2-bs1, AN2-bs2 and AN2-bs3) and a negative control with a low predicted binding score (AN1-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 AN1-bs1, AN1-bs2 or AN2-bs3 DNA fragments, whose position is depicted in (C-E). Similar results were obtained in 5 additional independent assays for AN1-bs1, 2 additional independent assays for AN2-bs3 and 4 additional independent assays for AN1-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, AN1 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 ± s.e.m.


