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RESEARCH ARTICLE

Non-cell-autonomous regulation of petal initiation in *Arabidopsis thaliana*

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ABSTRACT

In many flowering plants, petals initiate in alternate positions from first whorl sepals, suggesting possible signaling between sepal boundaries and petal initiation sites. *PETAL LOSS* (*PTL*) and *RABBIT EARS* (*RBE*) regulate petal initiation in *Arabidopsis thaliana* and their transcripts are expressed in sepal boundary and petal initiation sites, respectively, suggesting that *PTL* acts in a non-cell-autonomous manner. Here, we determined that cells expressing PTL and RBE fusion proteins did not overlap but were adjacent, confirming the non-cell-autonomous function of PTL. Genetic ablation of intersepal cells by expressing the diphtheria toxin-A chain gene driven by the *PTL* promoter resulted in flowers lacking petals, suggesting these cells are required for petal initiation. Transcriptome analysis combined with a PTL induction system revealed 42 genes that were upregulated under PTL activation, including *UNUSUAL FLORAL ORGANS* (*UFO*), which likely plays an important role in petal initiation. These findings suggest a molecular mechanism in which PTL indirectly regulates petal initiation and *UFO* mediates positional signaling between the sepal boundary and petal initiation sites.

KEY WORDS: *Arabidopsis thaliana*, *PETAL LOSS* (*PTL*), Petal primordia, *RABBIT EARS* (*RBE*), Sepal boundary, *UNUSUAL FLORAL ORGANS* (*UFO*)

INTRODUCTION

Flower morphology is important for attracting pollinators, such as flying insects or birds. The arrangement of floral organs in floral buds is roughly classified into two patterns: the spiral pattern, observed in several clades, including basal angiosperms (Endress, 2001; Endress and Doyle, 2007); and the whorled (or concentric) pattern seen in many other flowering plants. Flowers generally consist of four types of floral organ: sepals or outer tepals; petals or inner tepals; stamens; and carpels. In whorled flowers, different organ identities in each whorl of the flower are specified by distinct complexes of floral homeotic proteins. This regulation is explained by the floral ABC model or the more advanced ABCE model, which both describe the genetic mechanism for the establishment of floral identity in most flowering plants (Theissen et al., 2016).

Floral organs generally arise in a more or less equally spaced pattern within the whorl. In *Arabidopsis thaliana*, two medial sepals arise on the adaxial and abaxial sides of the inflorescence meristem, followed by two sepals at the lateral positions at floral stage 3 (Smyth et al., 1990). The second whorl contains petals, which usually occur in alternate positions to the sepals (or outer tepals). This suggests that positional information for the intersepal region is transmitted to the inner petal founder cells to fix the organ positions within the second whorl. However, the molecular mechanism for this positional signaling remains unknown.

Genetic approaches have identified regulators involved in the early development of second whorl organs in *Arabidopsis*. The *petal loss* (*ptl*) mutant has defects in sepal separation and petal initiation (Griffith et al., 1999). *PTL* encodes a GT2-clade trihelix transcription activator and is expressed in intersepal cells. These cells do not overlap with cells expressing the auxin-responsive DR5 reporter gene, suggesting that PTL regulates petal development indirectly (Brewer et al., 2004; Lampugnani et al., 2012). *RABBIT EARS* (*RBE*) encodes a C2H2-type zinc finger protein and its mutation results in the deformation or elimination of petals (Takeda et al., 2004). *RBE* is expressed in petal primordia cells, in which it negatively regulates the transcription of *AGAMOUS*, *MIR164*, *TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR 4* (*TCP4*), *TCP5* and *PUB25* to facilitate the early development of petal primordia (Huang and Irish, 2015; Huang et al., 2012; Krizek et al., 2006; Li et al., 2016, 2021). When the *ptl* or *rbe* mutant was combined with the homeotic mutant *apetala3* (*ap3*), the second and third whorl organs of which are replaced by sepals and carpels, respectively, the double mutants showed a reduced number of sepalloid second whorl organs (Griffith et al., 1999; Takeda et al., 2004), suggesting that *PTL* and *RBE* are involved in the development of second whorl organs independently of organ identity.

Several pieces of evidence suggest that *PTL* and *RBE* regulate petal initiation in the same pathway: first, both mutants show similar petal-loss phenotypes; second, the *ptl rbe* double mutant resembles the *ptl* single mutant (Lampugnani et al., 2013); and third, *RBE* expression is absent in the petal primordia of the *ptl* mutant (Takeda et al., 2004). These findings suggest that positional signal transduction occurs between the sepal boundary, where *PTL* is expressed, and the petal primordium, where *RBE* is expressed.

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Here, we uncovered a molecular link between the sepal boundary and petal initiation site and the role of this link in petal initiation. Using fluorescent fusion proteins, we examined the spatiotemporal expression patterns of PTL and RBE in floral buds. PTL was detected in sepal boundaries and RBE was detected in petal initiation sites; the fluorescent signals were adjacent but not overlapping, confirming the notion that PTL regulates petal primordium initiation indirectly. We used the glucocorticoid induction system to enable temporal activation of *PTL* and determined that several genes were activated by PTL, including *UNUSUAL FLORAL ORGANS* (*UFO*). The *ptl ufo* double mutant resembled the *ptl* single mutant, suggesting that UFO acts downstream of PTL. Our findings shed light on the signal transduction of positional information between the sepal boundary and petal initiation sites, which is required for early petal development.

RESULTS

PTL regulates petal initiation in a non-cell-autonomous manner

Previous studies showed that *PTL* is expressed in sepal boundary cells, whereas the *ptl* mutant either lacks, or has deformed, petals, suggesting that *PTL* regulates petal initiation in a non-cell-autonomous manner (Brewer et al., 2004; Griffith et al., 1999). To investigate whether PTL moves from intersepal cells to petal initiation sites, we generated a translational fusion of PTL and CFP expressed by the *PTL* own promoter and terminator (*PTLg:CFP*; Fig. S1). The construct restored petal development in *ptl-1* and the fluorescent signal remained at intersepal cells (Fig. 1A,E,I), suggesting that the fusion protein was functional and that PTL functions in a cell-autonomous manner at the sepal boundary.

To elucidate whether the PTL-expressing region contains cells that give rise to petals, we generated *GFP:RBEg PTLg:CFP* plants. *RBE* is transcribed and translated in petal primordium cells and is essential for petal primordium initiation (Fig. 1B; Takeda et al., 2014). The expression domains of PTL and RBE did not overlap but were adjacent (Fig. 1C,D; Movies 1 and 2), indicating that the domains of the intersepal and petal initiation sites were neighboring but did not overlap.

To confirm the PTL expression at the sepal boundary, we crossed *PTLg:CFP* plants with a line expressing fluorescently labeled proteins in boundary domains. *CUP-SHAPED COTYLEDON1* (*CUC1*) and *CUC2* are expressed in the organ boundaries in the aerial organs of plants and regulate boundary identity (Aida et al., 1997; Ishida et al., 2000; Takada et al., 2001). The translational fusion of CUC1 and GFP in *CUC1g:GFP* plants was expressed in the boundary of the sepal and floral meristems (Fig. 1F), whereas the CUC2-GFP fusion protein in *CUC2g:GFP* plants was expressed in a broader region compared with CUC1 (Fig. 1J). Plants carrying both *PTLg:CFP* and *CUC1g:GFP* contained a few cells in which both signals were detected at the sepal boundary (Fig. 1G,H; Movie 3). By contrast, *PTLg:CFP CUC2g:GFP* plants contained more cells expressing both PTL and CUC2 at the sepal boundary (Fig. 1K,L; Movie 4). These data support the notion that *PTL* is transcribed and translated in the sepal boundary and, thus, regulates petal primordium development in a non-cell-autonomous manner.

PTL-expressing cells are required for petal initiation and development

To examine whether sepal boundary cells are required for petal initiation, we genetically ablated these cells using diphtheria toxin A chain (DT-A) (Bellen et al., 1992). DT ribosylates the EF2

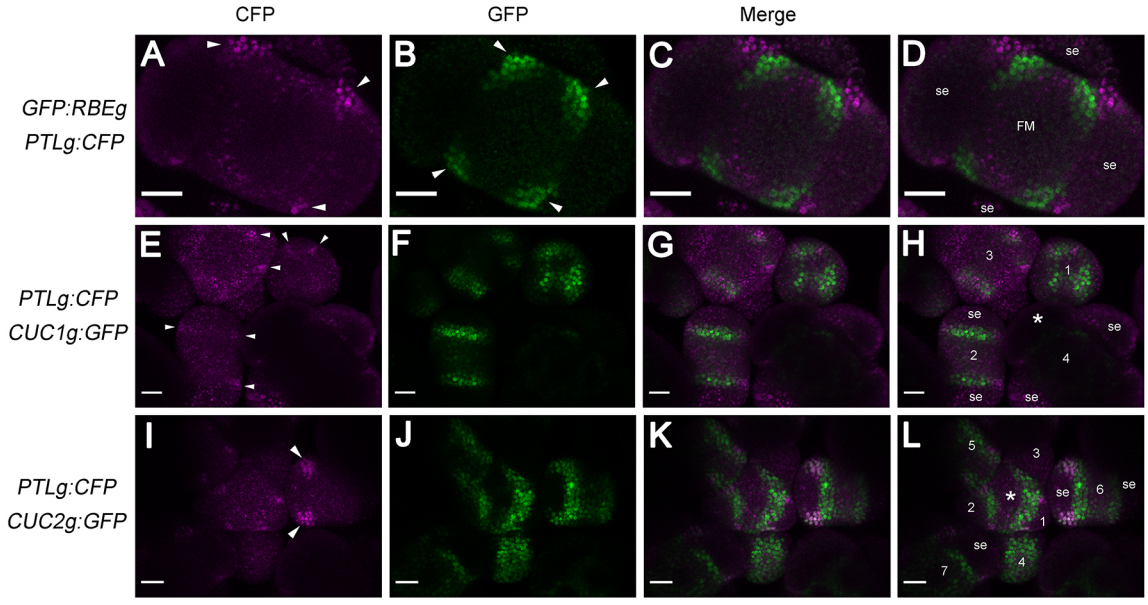

Fig. 1. Expression of translational fusion proteins in sepal boundaries and petal initiation sites. (A,E,I) CFP signals are in magenta; (B,F,J) GFP signals are in green. (C,G,K) Merged images of CFP and GFP signals. (D,H,L) Same as C,G,K with labels. Asterisks indicate the shoot apical meristem (SAM); numbers in H and L indicate the order of the floral meristem (FM) arising from the SAM. (A-D) Top view of a stage 3 flower bud from a *GFP:RBEg PTLg:CFP* plant. (A) PTL expression at the sepal boundary (arrowheads). (B) RBE expression in petal initiation sites (arrowheads). (C,D) PTL and RBE expression zones are adjacent but do not overlap. (E-H) Top views of *PTLg:CFP* and *CUC1g:CFP* inflorescences. (E) PTL expression at sepal boundaries (arrowheads). (F) CUC1 expression at the boundary of the FM and sepal. CUC1 is also expressed in earlier FM. (G) PTL and CUC1 expression zones overlap in a few cells at the sepal boundary. (I-L) Top views of *PTLg:CFP* and *CUC2g:GFP* inflorescences. (I) PTL expression at the sepal boundary (arrowheads). (J) CUC2 expression at the boundaries of the SAM-FM and FM-sepal regions. (K) PTL expression zone overlaps more broadly with CUC2 than with CUC1. Scale bars: 20 µm. se, sepal primordia.

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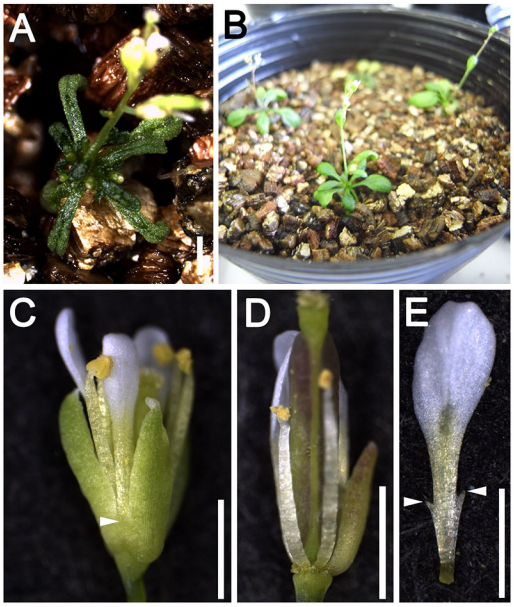

Fig. 2. Developmental defects of *PTLg:DTA* plants. (A,B) Severe lines with narrow, dark-green leaves (A) and a few flowers (B). (C-E) Flower phenotypes of mild lines. (C) Sepals fail to separate at the base (arrowhead). (D) Petal does not initiate. Sepals were removed to show the inside of the flower. (E) Two serrations occur in the proximal part of the petal (arrowheads). Scale bars: 1 mm.

translation initiation factor and inhibits protein synthesis, causing cell death. DT-A is a component of DT that kills cells in a cell-autonomous manner and has been used for tissue-specific genetic ablation in plants (Day et al., 1995; Nilsson et al., 1998; Takeda et al., 2004; Tsugeki and Fedoroff, 1999). We generated *PTLg:DT-A* plants in the wild-type (Colombia-0; Col-0) background. Among the 24 lines isolated by screening in the T1 generation, nine lines showed a small stature, with narrow, dark-green leaves, and subsequently developed a few flowers (Fig. 2A). Narrow leaves suggest cell death in the edge region, where *PTL* is expressed (Brewer et al., 2004). Eleven other lines were small, with small, green leaves and set of flowers with reduced numbers of petals and stamens (Fig. 2B; Table S2). The other two lines showed normal vegetative growth and set seeds, and their offspring generated flowers with fused sepals (Fig. 2C) and fewer petals compared with Col-0 (Fig. 2D), resembling the phenotypes of the *ptl* mutant. Some petals exhibited serration at their proximal regions (Fig. 2E). These results indicate that genetic ablation of *PTL*-expressing cells affects sepal separation, petal initiation and petal morphology, confirming the notion that the sepal boundary is required for petal initiation.

Downstream genes regulated by PTL

To identify genes involved in positional signal transduction, we examined genes regulated by PTL. We developed a chemical induction system of *PTL* using the rat glucocorticoid receptor (GR; Aoyama and Chua, 1997). A translational fusion of PTL and GR was expressed under the control of the *PTL* promoter and terminator (*PTLg:GR*) in the *ptl-1* mutant background (Fig. S1). First, we applied mock or DEX solution onto each inflorescence once daily. The mock treatment did not restore petal formation in the mutant (Fig. 3A), whereas DEX-treated inflorescences developed petals after 7-10 days (Fig. 3B), indicating that the PTL-GR fusion protein was functional. Next, to determine whether the temporary action of PTL is sufficient for petal initiation, we applied mock or DEX treatment to inflorescences only once, finding that petals developed after 10 days in DEX-treated plants but not in mock-treated plants (Fig. 3C,D). According to the floral stages defined by Smyth et al. (1990), an open flower at stage 13 was at stage 5 (bud) 10 days earlier, when petal and stamen primordia arose. Therefore, when flowers at the petal initiation stage (i.e. around stage 5) were induced to express *PTL* by DEX treatment, they successfully initiated petal primordia. This further suggests that, once *PTL* is activated, subsequent stages of petal development progress to the final steps in morphogenesis. Our data highlight the importance of PTL as a master regulator of petal initiation and development.

Using this induction system combined with RNA-sequencing analysis, we identified genes that function downstream of PTL. We identified 42 upregulated and seven downregulated genes in the inflorescences 3 h after DEX treatment compared with mock control plants (Fig. 3E; Table 1). Among the upregulated genes, we focused on the F-box gene *UNUSUAL FLORAL ORGANS* (*UFO*)/*At1g30950*; the upregulation of this gene was confirmed by RT-qPCR (Fig. 3F). These results suggest that *UFO* plays a major role in petal development under PTL regulation. We also examined *UFO* expression in *PTLg:GR* plants by mRNA *in situ* hybridization. *UFO* is expressed in the center of the floral primordium at stage 2, in the cup-shaped domain during stage 3 and in four clusters at the base or abaxial side of the petal primordium at stage 4 (Durfee et al., 2003; Ingram et al., 1995; Laufs et al., 2003; Lee et al., 1997; Samach et al., 1999). In mock-treated plants, *UFO* was expressed in the cup-shaped domain at stage 3 (Fig. 4A) but was absent from four clusters at later stages (Fig. 4B). By contrast, in DEX-treated inflorescences, *UFO* was expressed in four clusters at stages 4 and 5 (Fig. 4C,D), supporting the notion that PTL regulates *UFO* expression specifically in these four clusters.

To confirm the genetic relationship between *PTL* and *UFO*, we generated *ptl ufo* double mutants. We used a weak *ufo* allele, *ufo-6*, to examine the effects of *ptl* on petal development (Lee et al., 1997; Levin and Meyerowitz, 1995). Flowers of the *ptl-1 ufo-6* double mutant resembled those of the *ptl-1* single mutant (Fig. 4E-H), which is consistent with our hypothesis that *UFO* acts downstream of *PTL*.

At1g11620, encoding an F-box protein in a family distinct from UFO (Kuroda et al., 2002), was also highly upregulated in DEX-treated *PTLg:GR* plants (Table 1; Fig. 3G). We examined the T-DNA insertion mutant for this gene (SALK_068307) but did not detect any differences from the wild type in whole plants, including flowers. Therefore, whether this gene is involved in petal initiation remains unknown.

Interestingly, *TDF1/At3g28470* was also upregulated in DEX-treated *PTLg:GR* plants (Table 1; Fig. 3H). *TDF1* encodes an R2R3 MYB transcription factor that affects late tapetal function and subsequent pollen development (Zhu et al., 2008). *TDF1* expression is first detected in anthers at anther stage 5, corresponding to flower stage 9 (Sanders et al., 1999; Smyth et al., 1990; Zhu et al., 2008). *PTL* is expressed in lateral regions of the stamen primordium at floral stages 7-9 (Brewer et al., 2004). Although their expression patterns need to be examined in detail, it is possible that PTL activates *TDF1* expression in the stamen primordium.

DISCUSSION

PTL is suggested to regulate petal initiation in a non-cell-autonomous manner and we confirmed this here by protein localization at the sepal boundary. Using a DEX induction system and transcriptome analysis, we identified 42 upregulated genes in DEX-treated *PTLg:GR* plants versus controls. Among these, we

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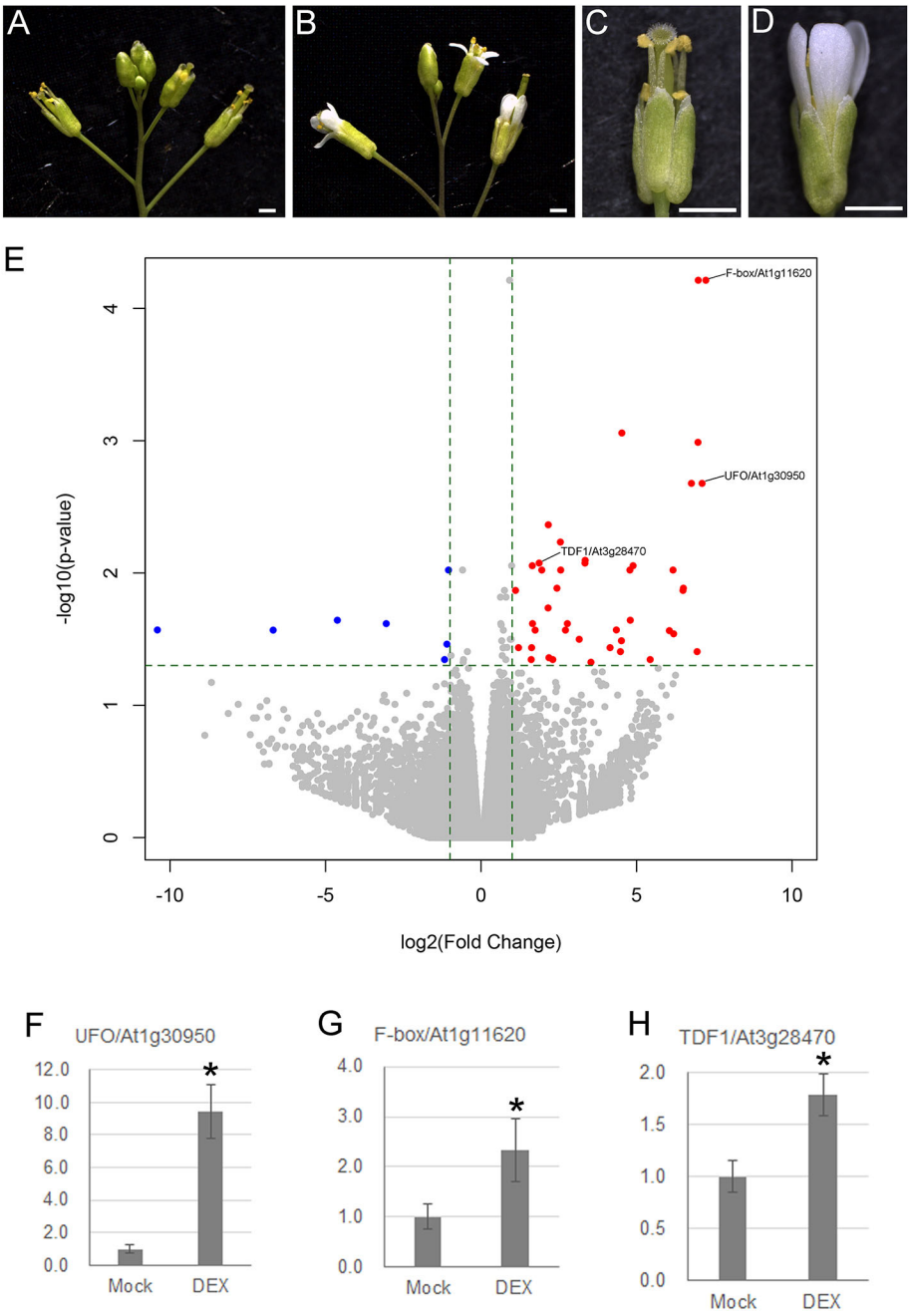

Fig. 3. Transcriptome analysis of downstream genes of PTL. (A-D) *PTLg:GR* plants. (A,C) Mock-treated plants. (B,D) DEX-treated inflorescence, restoring petal development. (E) Volcano plot showing the expression profiles of up- or downregulated genes in DEX-induced *PTLg:GR* plants. Genes that showed significantly altered expression after 3 h of treatment were included in the plot. The three genes selected for RT-qPCR analysis are indicated. (F-H) RT-qPCR analysis of up- or downregulated genes. Expression was normalized by *AtTUB4*. Values represent the mean±s.d. **P*<0.05 (Student's *t*-test, *n*=3). Scale bars: 1 mm.

focused on the role of *UFO* in floral organ development. *UFO* was expressed in four clusters upon PTL induction (Fig. 4). The *ptl-1 ufo-6* double mutant resembled the *ptl-1* single mutant (Fig. 4), and *ufo* is epistatic to *rbe-3* in the development of the second whorl organs (Krizek et al., 2006), suggesting that UFO mediates positional signaling between PTL and RBE.

UFO, an F-box protein belonging to the SCF ubiquitin complex, regulates multiple processes during floral organ development (Ingram et al., 1995; Laufs et al., 2003; Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995). One of the major roles of UFO is to activate class B homeotic genes, especially *APETALA3* (*AP3*), with other components of the SCF complex (Lee et al., 1997; Ni et al., 2004; Samach et al., 1999; Wang et al., 2003; Wuest et al., 2012; Zhao et al., 2001). The activation of *AP3* is mediated by the direct interaction of UFO with LFY, which directly binds to the *AP3* promoter for transcriptional activation (Chae et al., 2008). AP3 binds to the terminator region of *RBE* and regulates its transcription (Wuest et al., 2012), suggesting that UFO is involved in activating *RBE* via AP3 activity. However, because *AP3* is expressed in a whorled pattern, whereas *RBE* expression is restricted to the petal primordia (Takeda et al., 2004), another mechanism must exist for the positioning of gene expression to the organ primordia in the second whorl.

The other function of UFO is to regulate petal initiation: some *ufo* alleles result in flowers lacking petals (Durfee et al., 2003). UFO is expressed in floral meristems at stage 1, in three inner whorls at stage 2, in a cone-shaped region at stage 3, in four clusters lying on the abaxial side of the petal primordia at stage 4 and in petal primordia from stages 5 to 6 (Durfee et al., 2003; Ingram et al., 1995; Laufs et al., 2003). Therefore, UFO is likely involved in the position-dependent initiation of petal primordia at stage 4.

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Table 1. Differentially expressed genes in DEX- versus mock-treated *PTLg:GR* plants

AGI code	Annotation	Length (aa)	Sum	Mock_03h	DEX_03h	DEGs03h_logFC	DEGs03h_FDR
Upregulated genes							
AT1G11620	F-box	363	6.29	0	1.21	7.23	0.0001
AT1G30950	UFO/F-box	442	3.30	0	1.11	7.11	0.0021
AT4G36610	Alpha/beta-hydrolase superfamily	317	6.44	0	1.02	6.98	0.0001
AT5G28210	mRNA capping enzyme family	625	3.75	0	1.01	6.97	0.0010
AT1G49952	Unknown	-	3.26	0	0.99	6.95	0.0393
AT5G49370	Pleckstrin homology domain	100	4.66	0	0.88	6.76	0.0021
AT2G04840	F-box only protein (DUF295)	389	2.43	0	0.73	6.51	0.0130
AT5G60130	AP2/B3-like transcription factor family protein	326	4.35	0	0.72	6.49	0.0135
AT1G45100	RNA-binding (RPM/RBD/RNP motifs) family	497	2.83	0	0.58	6.19	0.0288
AT3G62780	Calcium-dependent lipid-binding (CaLB domain) family	298	3.43	0	0.58	6.17	0.0095
AT1G15010	Mediator of RNA polymerase II transcription subunit	142	2.73	0	0.53	6.05	0.0273
AT1G12030	Phosphoenolpyruvate carboxylase, putative (DUF506)	295	4.27	0	0.35	5.44	0.0451
AT3G51560	TIR-NBS-LRR family	1253	4.57	0.03	0.97	4.89	0.0088
AT3G11920	Glutaredoxin-like protein	630	3.65	0.03	0.91	4.80	0.0227
AT1G55380	Cys/His-rich C1 domain	661	4.81	0.03	0.90	4.79	0.0095
AT5G07620	Protein kinase superfamily	359	8.48	0.05	1.33	4.53	0.0009
AT3G21120	F-box	367	3.87	0.02	0.75	4.51	0.0325
AT1G67020	Transmembrane protein	659	5.05	0.03	0.73	4.48	0.0393
AT1G17240	AtRLP2/CLV2-related	729	3.15	0.03	0.67	4.35	0.0269
AT2G28990	LRR kinase family	884	4.43	0.05	1.02	4.15	0.0367
AT1G64800	DNA binding/TF	115	11.83	0.05	0.67	3.53	0.0473
AT2G35890	CPK25/calcium-dependent protein kinase	520	6.41	0.12	1.34	3.35	0.0080
AT5G28320	Unknown	927	15.91	0.28	2.87	3.34	0.0084
AT1G26515	F-box	366	7.90	0.25	2.30	3.16	0.0317
AT3G28640	Tetratricopeptide repeat (TPR)-like superfamily	504	22.80	0.53	3.65	2.78	0.0241
AT5G15340	Pentatricopeptide repeat (PPR) superfamily	623	10.93	0.25	1.69	2.71	0.0270
AT1G71490	TPR-like superfamily	681	29.33	0.68	4.03	2.56	0.0095
AT4G18490	Unknown	756	19.86	0.65	3.86	2.55	0.0058
AT1G17232	Potential natural antisense genes	-	18.35	0.60	3.30	2.44	0.0130
AT3G46370	LRR protein kinase family	793	7.94	0.35	1.76	2.31	0.0451
AT1G68930	PPR/pentatricopeptide repeat-containing	743	22.79	0.85	3.90	2.18	0.0437
AT5G52290	SHORTAGE IN CHIASMATA 1/XPF endonuclease	1594	18.16	0.75	3.40	2.16	0.0043
AT1G06310	ACX6/putative acyl-CoA oxidase	675	12.74	0.68	3.05	2.16	0.0184
AT1G67370	Meiotic asynaptic mutant 1	596	24.15	0.97	3.79	1.95	0.0095
AT3G28470	AtMYB35/TDF1 (anther morphogenesis)	317	25.94	1.20	4.40	1.87	0.0084
AT2G40250	SGNH hydrolase-type esterase superfamily	361	24.07	1.08	3.62	1.74	0.0270
AT2G12490	Transposable element	-	27.98	1.38	4.35	1.66	0.0241
AT5G25950	Unknown/DUF239	432	41.68	2.31	7.24	1.65	0.0088
AT1G17960	Threonyl-tRNA synthase	458	19.84	0.93	2.87	1.63	0.0367
AT3G62200	Putative endonuclease or glycosyl hydrolase	673	74.01	3.65	11.17	1.61	0.0451
AT2G20810	GAUT10/LGT4, galacturonosyltransferase 10	536	105.58	7.05	16.27	1.21	0.0367
AT1G30420	ABC transporter C11	1495	101.52	8.23	17.75	1.11	0.0135
Downregulated genes							
AT3G23170	Unknown	107	153.08	31.91	15.45	−1.05	0.0095
AT5G63180	Pectin lyase-like superfamily	432	75.78	9.51	4.44	−1.10	0.0345
AT2G43230	Protein kinase superfamily	440	88.60	14.82	6.57	−1.17	0.0451
AT2G38940	AtPT2/T phosphate transporter 1;4	534	7.24	1.00	0.11	−3.05	0.0241
AT4G32280	IAA29	251	2.72	0.75	0.02	−4.62	0.0227
AT5G46871	Defensin-like family	78	1.45	0.83	0.00	−6.68	0.0270
AT2G39060	AtSWEET9	258	14.44	11.01	0.00	−10.41	0.0269

*3 h DEX versus mock, FDR<0.05, $\log_2$FC <−1 or >1.

We propose a genetic mechanism for petal initiation in the second whorl involving two pathways: a pathway for the determination of organ identity in a whorled pattern; and a pathway for organ positioning (Fig. 5; Fig. S2; Table S3). LFY and UFO activate class B genes, which establish the petal identity together with class A genes in the second whorl. Simultaneously, *PTL* is activated in the sepal boundary region, likely by CUC1, given that transcriptome analysis showed that *PTL* is upregulated in *35S:CUC1* plants (Takeda et al., 2011). PTL expressed in the sepal boundary then activates *UFO*, and UFO activates *RBE* in the petal primordia. We examined *UFO* expression in *PTLg:GR* plants 3 h after treatment with cycloheximide and DEX treatment but did not detect its expression. Moreover, it appears, although not definitely so, that four clusters in which *UFO* is expressed at stage 4 include intersepal cells; thus, whether *UFO* is a direct transcriptional target of PTL remains unknown.

There may be crosstalk between these two pathways, because AP3 directly activates *UFO* and *RBE* (Wuest et al., 2012), and RBE suppresses class B genes (Takeda et al., 2014). The latter negative feedback regulation might enable temporal expression of *RBE* during petal initiation. Together, these signals might be integrated and lead to the initiation of petals at alternate positions from sepals in the second whorl at floral stage 5. Given that UFO is a component of the SCF complex, we propose that a protein degradation process

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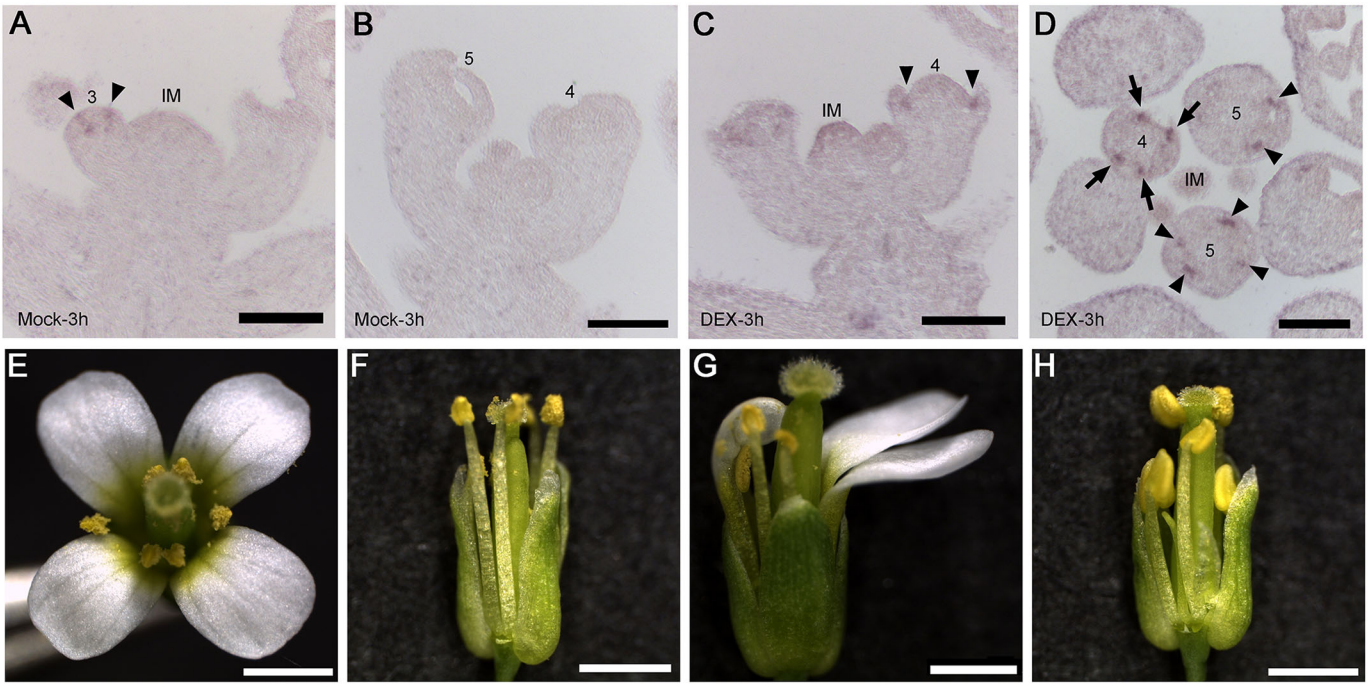

Fig. 4. Expression and genetic relationship of *UFO* and *PTL*. (A,B) *UFO* expression in mock-treated *PTLg:GR* inflorescences. *UFO* is expressed in the cup-shaped domain at stage 3 (A, arrowheads) but not in petal primordia at a later stage (B). (C,D) *UFO* expression in DEX-treated *PTLg:GR* inflorescences. *UFO* is expressed in four clusters at stage 4 (arrows) and petal primordia at stage 5 (arrowheads). Numbers indicate floral stages. (A-C) Longitudinal sections. (D) Transverse section. (E-G) Flower phenotypes of *ptl* and *ufo* mutants: (E) *Ler*, (F) *ptl-1*, (G) *ufo-6* and (H) *ptl-1 ufo-6* double mutant. Front sepals were removed in F and H. Scale bars: 100 µm in A-D; 1 mm in E-H. IM, inflorescence meristem.

occurs during petal initiation. Indeed, several F-box genes were upregulated in DEX-treated *PTLg:GR* plants (Table 1), suggesting that proteolysis is an important process for position-dependent petal initiation.

PTL is thought to promote auxin accumulation at petal initiation sites, and auxin-related factors, such as *AUX1*, *PID* and *PIN*, act downstream of PTL (Lampugnani et al., 2013). Expression of *AUX1* at intersepal cells driven by the control of the *PTL* promoter restored petal development in the *ptl* mutant, suggesting that auxin biosynthesis at intersepal cells is required for petal initiation. However, in this study, we did not identify any upregulated auxin-related genes under PTL induction (Table 1). *IAA29*, an auxin-responsive gene, was downregulated in DEX-treated *PTLg:GR* plants based on transcriptome data, but RT-qPCR analysis showed that this difference was not significant (Table 1; Fig. S3). Therefore, although the PTL-dependent auxin pathway is crucial for petal initiation, we suggest that this type of regulation occurs later than the early response of transcriptional regulation by PTL.

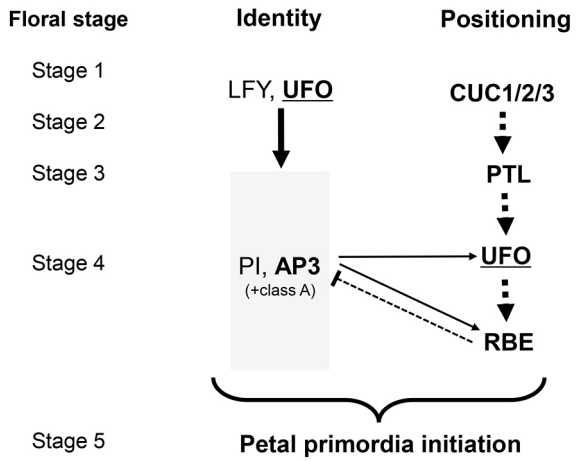

Fig. 5. Model of the genetic pathway for petal primordia initiation. Solid lines show direct regulation, and dashed lines indicate either direct or indirect regulation. At floral stage 1 to 2, LFY and UFO activate the class B genes *AP3* and *PI*, establishing organ identity in the second and third whorls. Simultaneously, *PTL* is activated by CUC1/2/3 at the sepal boundary. PTL activates *UFO* at the sepal boundary and transmits the positional signal to *RBE*, which is expressed in petal primordia. There may be crosstalk between these two pathways because AP3 activates *UFO* and *RBE* expression and RBE suppresses *AP3* expression.

The differentially expressed genes in PTL-induced plants included several genes encoding transcription factors, secondary metabolic enzymes and transporters (Table 1), but we did not identify genes that were reported to be involved in petal development. Given that *PTL* is expressed not only in the sepal boundary during early stages of flower development, but also in the lateral domains of sepals and petals (Brewer et al., 2004), the differentially expressed genes may include genes involved in the development of the lateral regions of perianth organs, such as *TDF1* (involved in stamen development), as shown above.

In conclusion, we suggest that the F-box protein UFO mediates the signaling of positional information between the sepal boundary (PTL) and petal initiation sites (RBE). This idea might explain how PTL regulates petal initiation in a non-cell-autonomous manner and why petals are located at alternate positions from sepals. *UFO* orthologs are conserved in other flowering plants, including *Antirrhinum* and pea (Ingram et al., 1997; Taylor et al., 2001; Wilkinson et al., 2000). In *Antirrhinum*, the orthologous gene *fimbriata* (*fim*) is involved in the specification of organ positioning and the maintenance of organ boundaries (Ingram et al., 1997). This

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observation suggests that non-cell-autonomous regulation of petal initiation is conserved in flowering plants, a process mediated by a boundary gene, an F-box gene and a petal primordium gene.

MATERIALS AND METHODS

Plant growth conditions

A. thaliana plants were grown in vermiculite in small pots under long-day conditions (16 h light/8 h dark) at 23-25°C. *Arabidopsis* ecotypes Col-0 and Landsberg *erecta* (Ler) were used as the wild types. The *ptl-1*, *rbe-1* and *ufo-6* mutants were from laboratory stocks, which were originally obtained from a stock center (https://abrc.osu.edu/; *ufo-6*) or were a gift from Prof. David Smyth (Monash University, Melbourne, Australia; *ptl-1*).

Plasmid construction and transformation

The *PTL* constructs generated in this study are shown in Fig. S1. To generate genomic fusions of PTL with Cyan Fluorescent Protein (SECFP), a 6.3-kb genomic fragment that included the *PTL* promoter and the coding sequence without the stop codon was amplified from Col-0 using 5′-CGGGATCC-GATATCATTACGTGTTTGTCGGCA-3′ and 5′-GGGGTACCCTGATT-CTCTTCTTTACTGAGCCT-3′ primers, digested by BamHI and KpnI, and ligated to pAN19 to generate pPTLpg19. The 1.2-kb *PTL* terminator was amplified using 5′-GGAGTCGAGCTCGTAATTTCTCTTAATGAAGA-AGAA-3′ and 5′-CGGAATTCTCTAGACCAAATCAAGATCAAACA-3′ primers, digested by SacI and EcoRI, and cloned into pSECFP19, in which *SECFP* had been subcloned into the pAN19 vector, to generate pPTLtSECFP19. The BamHI and KpnI fragment from pPTLpg19 was subcloned into pPTLtSECFP19 to generate PTLp:PTLg::SECFP::PTLt (designed as PTLg:CFP). The PTLg:GR plasmid was constructed in the same manner, except that *GR* was used, which was amplified using 5′-GGGGTACCCAGCAAGCCACTGCAGGAGTC-3′ and 5′-GGAGTC-GAGCTCTCATTTTTGATGAAACAGAAG-3′ primers, digested by KpnI and SacI, and cloned into pAN19. For PTLg:DTA, the *SECFP* region of PTLgCFP was replaced by the gene encoding DT-A, which was amplified using 5′-GGGGTACCATGGATCCTGATGATGTTGTT-3′ and 5′-CGC-GAGCTCTTAGAGCTTTAAATCTCTGTA-3′ and digested with KpI and SacI to generate PTLg:DTA. NotI fragments of PTLg:CFP, PTLg:GR, and PTLg:DTA were subcloned into the pBIN30 binary vector. For CUC2g: GFP, the *CUC2* promoter and coding sequence without the stop codon were amplified using 5′-GGGGACAACTTTGTATAGAAAAGTTGACTA-GAGGAAGAGTTAAGAGATG-3′ and 5′-GGGGACTGCTTTTTTGTA-CAAACTTGCGTAGTTCCAAATACAGTCAAG-3′ primers and cloned into pDONR P4-P1R using the Gateway system (Invitrogen). The *CUC2* terminator was amplified using 5′-GGGGACAGCTTTCTTGTACAA-AGTGGCATCACAAAAGAGGTGACTTATA-3′ and 5′-GGGGACAA-CTTTGTATAATAAAGTTGAAATCATCTAACCGAAGATTCG-3′ and cloned into pDONR P2R-P3 (Invitrogen). These two plasmids, together with pDONR207 carrying *GFP*, were transferred to the pGWB multisite vector to generate CUC2p:CUC2-GFP::CUC2ter (CUC2g:GFP). The plasmids were transformed into *Agrobacterium tumefaciens* (*Rhizobium radiobacter*) strain GV3101 (pMP90) and into Col-0 by the floral dip method (Clough and Bent, 1998). GFP:RBEg and CUC1g:GFP plants were generated as described previously (Gonçalves et al., 2015; Takeda et al., 2014). The plants carrying two constructs were generated by crossing, and their genotypes were checked by PCR.

Microscopy

For fluorescence microscopy, inflorescences were trimmed, mounted on glass slides with water and examined under an LSM780 confocal microscope (Carl Zeiss). Flower images were captured with an SPAP0 binocular microscope equipped with an EC3 digital camera system (Leica).

DEX treatment and expression analysis using the PTL induction system

The *PTLg:GR* construct was transformed into the *ptl-1* mutant. Twelve T1 lines showed resistance to 10 µg/ml glufosinate ammonium on selection medium (1% sucrose, 1% agar and 1×MS salt). Segregation and petal restoration were checked in the T2 generation. Seven lines showed restored petal development after treatment with DEX solution (10 µM dexamethasone in ethanol and 0.015% Silwet L-77). One homozygous line in the T3 generation was selected for further analysis. Mock treatment was performed with 0.1% ethanol and 0.015% Silwet L-77. After 3 h of DEX or mock treatment, total RNA was extracted from the inflorescences using an RNeasy Plant Mini Kit (QIAGEN). RNA integrity was confirmed using an Agilent RNA 6000 Nano Chip (Agilent Technologies). A library was constructed from a 0.5 µg RNA sample using an Illumina TruSeq Standard mRNA LT sample kit (Illumina). RNA sequencing was performed on the Illumina NextSeq 500 sequencing platform (Illumina), and data analysis was performed as described previously (Shimoki et al., 2021). We selected 42 and seven genes the expression of which was up- or downregulated, respectively, by summing those with a false discovery rate (FDR) <0.05, a sum (total number of mapped reads) >1, and a $\log_2$FC >1 (upregulated) or <−1 (downregulated).

For RT-qPCR, cDNA synthesis was performed using ReverTra Ace qPCR RT Master Mix with gDNA remover (Toyobo). The reaction was performed with Thunderbird SYBR qPCR mix (Toyobo) and a Dice Real Time System Lite thermal cycler (Takara Bio). The relative expression level was calculated based on amplification of the internal control gene *TUB4* (*At5g44340*). The primer sequences are listed in Table S1.

mRNA *in situ* hybridization

Inflorescences from *PTLg:GR* plants were treated with DEX or mock for 3 h and fixed with formalin/acetic acid/ethanol (FAA). mRNA *in situ* hybridization was performed as previously described (Takeda et al., 2004). The *UFO* antisense probe was generated from the pDW221.1 template (Lee et al., 1997) and labeled using a DIG RNA labeling kit (Merck).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: S.T.; Methodology: S.T., Y.H., T.S., S.K., M.A., T.H.; Software: Y.H., T.S., S.K.; Validation: S.T., T.S., S.K., M.A., T.H.; Formal analysis: S.T., S.K.; Investigation: S.T., Y.H., T.S., S.K., M.A.; Resources: S.T.; Data curation: S.T., Y.H., T.S., S.K.; Writing - original draft: S.T.; Writing - review & editing: S.T.; Visualization: S.T., Y.H., T.H.; Supervision: T.H.; Project administration: S.T.; Funding acquisition: S.T., S.K.

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Data availability

RNA-sequencing data are available in the DBJJ Sequenced Read Archive under the accession number DRA013479.

Peer review history

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