New Phytologist

Research

# LEAFY activity is post-transcriptionally regulated by BLADE ON PETIOLE2 and CULLIN3 in Arabidopsis

**Hicham Chahtane[1], Bo Zhang[2], Mikael Norberg[2], Marie LeMasson[1], Emmanuel Thévenon[1], László Bakó[3], Reyes Benlloch[2], Mattias Holmlund[2], François Parcy[1], Ove Nilsson[2] and Gilles Vachon[1]**

[1]Univ. Grenoble Alpes, CNRS, CEA, INRA, BIG-LPCV, 38054 Grenoble Cedex 9, France; [2]Umeå Plant Science Centre, Department of Forest Genetics and Plant Physiology, Swedish University of Agricultural Sciences, Umeå SE S-901 83, Sweden; [3]Umeå Plant Science Centre, Department of Plant Physiology, Umeå University, Umeå, Sweden

Authors for correspondence:
*François Parcy*
*Tel: +33 (0) 4 38 78 49 78*
*Email: francois.parcy@cea.fr*

*Ove Nilsson*
*Tel: +46 90 786 8487*
*Email: ove.nilsson@slu.se*

Received: *31 January 2018*
Accepted: *1 June 2018*

*New Phytologist* (2018)
**doi**: 10.1111/nph.15329

**Key words:** BLADE-ON-PETIOLE, cullin 3, flower development, inflorescence, LEAFY, post-transcriptional regulation, ubiquitination.

## Summary

- The *Arabidopsis* LEAFY (LFY) transcription factor is a key regulator of floral meristem emergence and identity. LFY interacts genetically and physically with UNUSUAL FLORAL ORGANS, a substrate adaptor of CULLIN1–RING ubiquitin ligase complexes (CRL1). The functionally redundant genes *BLADE ON PETIOLE1* (*BOP1*) and *-2* (*BOP2*) are potential candidates to regulate LFY activity and have recently been shown to be substrate adaptors of CULLIN3 (CUL3)–RING ubiquitin ligases (CRL3).
- We tested the hypothesis that LFY activity is controlled by *BOP*s and *CUL3*s in plants and that LFY is a substrate for ubiquitination by BOP-containing CRL3 complexes.
- When constitutively expressed, LFY activity is fully dependent on *BOP2* as well as on CUL3A and B to regulate target genes such as *APETALA1* and to induce ectopic flower formation. We also show that LFY and BOP2 proteins interact physically and that LFY-dependent ubiquitinated species are produced *in vitro* in a reconstituted cell-free CRL3 system in the presence of LFY, BOP2 and CUL3.
- This new post-translational regulation of LFY activity by CRL3 complexes makes it a unique transcription factor subjected to a positive dual regulation by both CRL1 and CRL3 complexes and suggests a novel mechanism for promoting flower development.

## Introduction

In *Arabidopsis*, the *LEAFY* (*LFY*) gene participates in floral meristem formation (Moyroud *et al.*, 2010; Chahtane *et al.*, 2013; Li *et al.*, 2013; Yamaguchi *et al.*, 2013) and regulates floral meristem identity by establishing, in particular, the expression of the MADS-box floral homeotic genes responsible for floral organ identity (Weigel & Nilsson, 1995; Blázquez *et al.*, 1997; Nilsson *et al.*, 1998; Parcy *et al.*, 1998; Parcy, 2005; Siriwardana & Lamb, 2012; Denay *et al.*, 2017). In *lfy* mutants, the inflorescence meristem fails to produce proper flowers and instead generates shoots or abnormal flowers subtended by bracts (Weigel *et al.*, 1992). Conversely, ectopic expression of LFY is sufficient to convert most aboveground meristems into flowers, demonstrating that the floral fate of meristems is governed by LFY (Weigel & Nilsson, 1995).

While *LFY* transcript and LFY protein are present throughout the floral meristem, direct target genes of LFY, such as the floral organ identity genes *APETALA1* (*AP1*), *APETALA3* (*AP3*), *AGAMOUS* (*AG*) or *SEPALLATA3*, are expressed in distinct spatial domains, implying that additional mechanisms are involved to confer distinct modes of action to LFY. One of these mechanisms is the concerted action of LFY with other partners, such as the transcription factors WUSCHEL (WUS) and SEPALLATA3, to activate *AG* (Lenhard *et al.*, 2001; Lohmann *et al.*, 2001; Liu *et al.*, 2009) or chromatin remodelling factors of the SWI2/SNF2 family (Wu *et al.*, 2012) that bind *AP3* and *AG* loci. Another LFY co-factor is the F-box protein UNUSUAL FLORAL ORGANS (UFO) required to activate *AP3* in Arabidopsis (Wilkinson & Haughn, 1995; Parcy *et al.*, 1998; Hepworth *et al.*, 2006; Chae *et al.*, 2008). In other species, such as petunia, rice or gerbera, the role of UFO appears wider, as it both triggers floral meristem growth and induces floral organ identity genes (Souer *et al.*, 2008; Zhao *et al.*, 2016). Such a broader role of UFO in floral meristem growth might also be valid for Arabidopsis, as plants mutated in both *UFO* and *FILAMENTOUS FLOWER* or *UFO* and *REVOLUTA* display aborted filaments instead of flowers (Sawa *et al.*, 1999).

F-box proteins are components of CULLIN1–RING (CRL1 or SKP–CULLIN1–F-Box (SCF)) complexes that act as E3 ubiquitin ligases to mark target proteins by ubiquitination, usually for proteasome-dependent degradation (Zhao *et al.*, 2001; Gagne *et al.*, 2002). CULLIN–RING ubiquitin ligase complexes (CRLs) E3 ligases constitute the largest superfamily of E3 complexes and share a similar modular architecture. The CULLIN (CUL) proteins (CUL1, CUL3 or CUL4 in plants) serve as an elongated

scaffold, holding the ubiquitin-conjugating E2 enzyme at one end and the substrate-receptor subunit at the other (Choi *et al.*, 2014). F-box proteins such as UFO are responsible for the specific recognition of the substrates by CRL1 complexes in which CUL1 is used as the scaffold. Studies in *Arabidopsis* and *Petunia* have shown a direct interaction between UFO and LFY (Chae *et al.*, 2008; Souer *et al.*, 2008). Moreover, the translational fusion between UFO and the SRDX repression domain mimics an *LFY* loss-of-function phenotype (Chae *et al.*, 2008), while a fusion between UFO and the VP16 activation domain produces ectopic meristems and flowers in an *LFY*-dependent manner (Risseeuw *et al.*, 2013). These results strongly suggest that complexes with LFY and UFO can occur at the DNA level and that UFO acts as a transcriptional co-factor to regulate LFY activity.

How the $SCF^{UFO}$ complex promotes LFY activity is unclear, but it might be similar to the activation of a variety of transcription factors in yeast or animals whose degradation by the ubiquitin proteasome system coordinately promotes their transcriptional activity (Kwak *et al.*, 2011; Shabek & Zheng, 2014). Although ubiquitinated forms of LFY were detected in overexpressing plants, they do not appear to be exclusively dependent on UFO activity, suggesting that LFY ubiquitination might depend on multiple E3 ligases (Chae *et al.*, 2008). As LFY is involved in the regulation of several temporally and spatially distinct gene expressions (Wagner *et al.*, 1999; Saddic *et al.*, 2006), such as *AP1* and *AG*, it is possible that LFY transcriptional activity would be differentially regulated by distinct interactions with E3 ligases complexes.

Among the genes involved in flower development, the functionally redundant genes *BLADE ON PETIOLE1* (*BOP1*) and *-2* (*BOP2*) are potential candidates to regulate LFY activity. BOP proteins are members of the small NON EXPRESSOR OF PATHOGENESIS RELATED GENES (NPR) family (see Khan *et al.*, 2014 for review) and are characterized by a BTB/POZ (for Broad Complex, Tramtrack, and bric-a-brac/POX virus and zinc finger) domain that is found in many proteins associated with the CULLIN3–RING E3 ligase (CRL3) complexes (Figueroa *et al.*, 2005; Weber *et al.*, 2005). BOP proteins are also known to be transcriptional co-activators (Jun *et al.*, 2010). It was recently shown that BOP proteins serve as substrate adaptors in a CUL3 E3 ligase complex to control the degradation of the PHYTOCHROME INTERACTING FACTOR4 (PIF4) (Zhang *et al.*, 2017). *BOP* and *LFY* genes display similar expression profiles in young floral meristems and participate in similar flower development processes, such as floral fate determinacy, bract suppression and meristem formation, although *BOP* genes are not restricted to these pathways and play additional roles, such as leaf margin development and abscission zone differentiation of floral organs (Norberg *et al.*, 2005; Karim *et al.*, 2009; Xu *et al.*, 2010). BOP was shown to regulate *AP1* expression independently of LFY (Xu *et al.*, 2010) and also to contribute to *LFY* expression together with PUCHI (Karim *et al.*, 2009).

In this study, we investigated whether BOP proteins are involved in controlling LFY activity and found that BOP2 could act as cofactor for the establishment of floral identity and *AP1* activation. We bring evidence that LFY is post-transcriptionally activated by *BOP2* and *CUL3A/B* genes in seedlings. Physical interactions between BOP2 and LFY and ubiquitination reactions *in vitro* using $CRL3^{BOP2}$ components support the hypothesis that a $CRL3^{BOP2}$ complex is necessary for LFY stability and transcriptional activity. The dual regulation of LFY by $CRL3^{BOP2}$ and $CRL1^{UFO}$ E3 ligases in a positive manner is a unique situation in plants and animals.

## Materials and Methods

### Plant materials and growth conditions

All *Arabidopsis thaliana* L. Heynh. (thale cress) plants used in this study are in Columbia-0 background. Seeds were sown on 1/2 Murashige and Skoog basal salt mixture medium, with addition of kanamycin for selection of plants DW151.2.5 containing the *35S::LFY* transgene (Weigel & Nilsson, 1995; Nilsson *et al.*, 1998). The *35S::LFY* transgene was homozygous in all lines except in the *cul3b*/+ background, for which no homozygous lines could be obtained. Petri dishes were kept 3 d at 4°C in the dark and transferred at 22°C under long day conditions (16 h : 8 h, light : dark). When required, seedlings were transferred on a soil–vermiculite (4 : 1 v/v) mixture. Mutant alleles used in this study have been characterized previously: *bop1-5* (SAIL T-DNA 14c02) and *bop2-2* (SALK T-DNA N575879) from Norberg *et al.* (2005); *cul3a-3* (SALK T-DNA 012973) and *cul3b-1* (GABY-KAT 003D02) from Thomann *et al.*, (2005); and *lfy-12* from Maizel & Weigel (2004). Oligonucleotides sequences used for plant genotyping are listed in Supporting Information Table S1. The 2.2 kb *AP1* promoter, which contains three LFY binding sites, was used for *AP1::GUS* experiments and was described previously (Benlloch *et al.*, 2011).

Phenotypic analysis described in Fig. 1 was done as follows. Plants were grown in long days, and nodes 1–40 of the primary inflorescence of each individual were characterized. For every five nodes, a mean was determined for each genotype using a majority rule. If more than half the individuals had bracts at a certain node position, a bract was indicated on that position in Fig. 1. If more than half of the bracts were larger than 2 mm then a large bract was indicated, otherwise a small bract was drawn.

### Western blots

Leaves from seedlings grown under long days in Petri dishes for 16 d (see later Figs 3, 8) or in soil for 4 wk (see later Fig. 4) were used for protein blot analysis. For each genotype, single leaves from three to five independent seedlings were pooled, weighed and frozen in liquid nitrogen to be powdered using an MM30 mixer (Retsch, http://www.retsch.com/) at 30 Hz for 2 min. Plant extracts were then suspended in denaturing buffer (50 mM trisaminomethane hydrochloride (Tris-HCl) pH 6.8; 1% sodium dodecyl sulfate (SDS); 10% glycerol; 0.4% dithiothreitol; 0.0025% bromophenol blue) with a ratio of 8 µl $mg^{-1}$ of initial fresh material. After 5 min at 95°C, 10 µl of plant extracts was separated using 10% SDS polyacrylamide gel electrophoresis and western blotted onto a polyvinylidene difluoride membrane

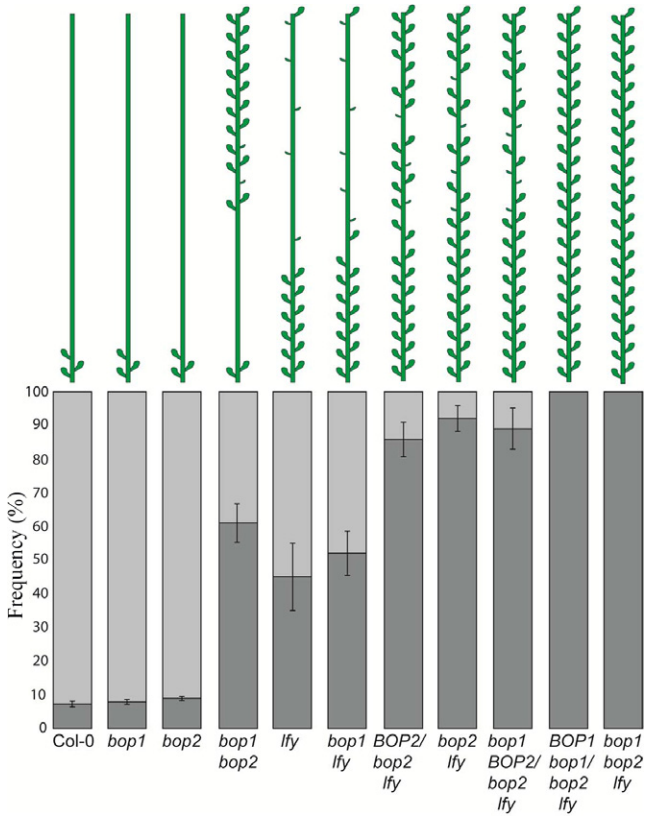


**Fig. 1** Cauline leaf, bract and shoot development in *Arabidopsis thaliana* plants with various combination of mutations. The upper panel shows schematic leaf/bract organization at the first 40 nodes of the main inflorescence axis. The first cauline leaf represents node 1. At each node, either a large cauline leaf/bract (> 2 mm), small cauline leaf/bract (< 2 mm) or absence of a cauline leaf/bract is shown. The lower panel shows frequency (%) of cauline leaf/bract development at the first 40 nodes of the main inflorescence. Dark grey shading represents the presence of cauline leaves/bracts; light grey represents absence of bracts. Error bars indicate the 95% confidence. Ten to 15 plants were analysed for each *A. thaliana* genotype. The null *lfy-12* allele was used (Maizel & Weigel, 2004).

(Immobilon®-P, Millipore http://www.merckmillipore.fr/). The remaining steps were done on Snap ID1 (Millipore) according to the manufacturer's instruction. Purified anti-LFY antibodies (named JA70 and raised in rabbit against recombinant LFY protein) were used as previously described (Moyroud *et al.*, 2011). Anti-KARI antibodies (described in Dumas *et al.*, 1989) or anti-RPN6 antibodies (BML-PW8370, Enzo Life Sciences, http://www.enzolifesciences.com/) were used as loading controls. Detection was done using an ECL2 western blotting substrate (Thermo Fisher Scientific-Life Technologies, Villebon sur Yvette, France) and scanned on a Typhoon 9400 scanner (GE Healthcare, Velizy Villacoublay, France) according to the manufacturer's instruction. LFY/Rubisco, LFY/KARI or LFY/RPN6 ratios were calculated for each genotype and using ImageJ software, with appropriate setup (uncalibrated optical density). Quantitative western blotting results presented here are then relative to wild-type control lane (%). Statistical analysis was done using R software (v.3.0.2).

## Bimolecular fluorescence complementation

For bimolecular fluorescence complementation assays, complementary DNA (cDNA) of LFY was introduced in pENTRY–LFY or pENTRY–LFY containing a STOP codon, and thus transferred by Gateway® Cloning (LR) into pBiFP destination vectors containing a split yellow fluorescent protein (YFP) coding sequence (Azimzadeh *et al.*, 2008). The newly formed vectors were then incorporated into *Agrobacterium tumefaciens* strain C58 pMP90 for tobacco transfection (namely pHC07, pHC08 and pHC09 coding respectively LFY–CYFP, LFY–NYFP and NYFP–LFY). cDNA BOP2 sequence was obtained by PCR from vector pGV732 and incorporated in a pENTRY–BOP2 or pENTRY–BOP2. The procedures followed were performed as described for LFY. Vectors introduced in *A. tumefaciens* strain C58 pMP90 are called pHC015 and pHC013 coding for BOP2–CYFP and NYFP–BOP2, respectively.

The LFY and BOP2 fusion constructs were co-transfected in four different combinations into 4-wk-old *Nicotiana benthamiana* leaves by agroinfiltration as previously described (Gehl *et al.*, 2009). Fluorescence of the lower epidermis of leaf discs 2 d after infiltration was visualized with a confocal microscope (Leica, Wetzlar, Germany). Lambda scans around the YFP emission were performed to ensure that the signal was a specific YFP signal.

## Quantitative PCR

RNA extractions were performed from isolated leaves of 16-wk-old seedlings grown in long days in Petri dishes using an RNA extraction kit (Qiagen, http://www.qiagen.com/) and treated with Ambion turbo DNA-free DNAse (Invitrogen, http://www.lifetechnologies.com/) to avoid genomic DNA contamination. Reverse transcription and quantitative PCR were performed as previously described (Chahtane *et al.*, 2013) except that *POLYUBIQUITIN10* (AT4G05320) was used as the reference gene. Primers used for real-time PCR were designed with the Quantprime software (www.quantprime.de/) and are listed in Table S1. PCR reactions were performed in 15 µl using 0.1 µM for each primer, 1× mix SYBR Green JumpStart® (Sigma-Aldrich) and 3.125 ng of cDNA in the Rotor Gene 3000 (Qiagen) with default cycle parameters. The relative expression of a target gene was calculated by the ΔΔCt method according to (Pfaffl, 2001) using the reference gene *POLYUBIQUITIN10* as previously described (Czechowski *et al.*, 2004; Guénin *et al.*, 2009). Statistical analyses were made using Rest 2009 software (Pfaffl *et al.*, 2002).

## Chromatin immunoprecipitation PCR

Whole 3-wk-old seedlings grown in Petri dishes were used. Plants were fixed at 4°C for 2 + 8 min in crosslinking buffer (1% formaldehyde, 0.4 M sucrose, 10 mM Tris-HCl pH 8, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA) under vacuum. The crosslink reaction was stopped by adding 0.1 M glycine under vacuum (4 + 4 min), then washed and frozen in liquid

nitrogen. Fixed frozen tissues were ground, and nuclear extraction was performed in Honda buffer (0.44 M sucrose, 1.25% Ficoll, 2.5% Dextran T40, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, potassium hydroxide pH 7.4, 10 mM magnesium chloride, 0.5% Triton X-100, 5 mM dithiothreitol, 1 mM PMSF, 1% plant protease inhibitors). Chromatin was extracted using nuclei lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS, 1 mM PMSF, 1% plant protease inhibitors) and sonicated with a Bioruptor® to obtain DNA fragments shorter than 300 bp. The solubilized chromatin was incubated with anti-LFY antibody JA-70 (Moyroud *et al.*, 2011) for 5 h at 4°C. Washed protein A agarose/salmon sperm DNA (Millipore) was incorporated in tubes and incubated overnight at 4°C under agitation. Beads were washed four times with binding/washing buffer (150 mM sodium chloride, 20 mM Tris-HCl pH 8.0, 2 mM EDTA, 1% Triton X-100, 0.1% SDS, 1 mM PMSF) followed by a washing using TE buffer (Tris 10 mM pH 8.0, EDTA 1 mM). Supernatant was removed and Chelex® 10% was added and samples were warmed 10 min at 95°C. Proteins were degraded by adding 20 µg proteinase K and incubated at 37°C for 1 h followed by inactivation of proteinase at 95°C. Supernatants were collected and used for PCR amplification of the *AP1* regulatory region containing the LFY binding site.

### *In vitro* ubiquitination assays

His-GST-LFY, His-GST-GFP, His-MBP-BOP2 and His-MBP-GFP (His, histone; GST, glutathione *S*-transferase; GFP, green fluorescent protein; MBP, myelin basic protein) were generated from *Escherichia coli* BL21 cells and purified using nickel nitrilotriacetic acid agarose from Qiagen according to the manufacturer's protocol. Human Cullin3/Ring-Box protein 1 (CUL3/RBX1) recombinant proteins were purchased from Ubiquigent (UK). The Neddylation of the Cullin3 was performed as previously described using a NEDD8 conjugation reaction buffer kit from R&D Systems Europe (Abingdon, UK) (Duda *et al.*, 2008). The recombinant human ubiquitin-activating enzymes (UBE1) and ubiquitin-conjugating enzymes (UbcH5b) were purchased from R&D Systems Europe. Ubiquitination reactions were performed as described previously with slight modification (Ni *et al.*, 2015). About 100 nM UBE1, 1 mM UbcH5b, 100 nM Cul3/Rbx1, 290 nM GST-LFY and 500 nM MBP-BOP2 were incubated at 30°C for 1 h in a buffer containing 40 mM biotin–ubiquitin, 5 mM adenosine 5′-triphosphate magnesium salt, 50 mM Tris-HCl pH7.6, 200 mM sodium chloride, 10 mM magnesium chloride, 1 unit inorganic pyrophophatase and 1 mM dithiothreitol. Reactions were then pulled down with 10 µl glutathione Sepharose 4B beads. GST-LFY and ubiquitinated proteins were detected by western blot analysis using anti-GST antibodies and streptavidin–horseradish peroxidase (HRP) conjugates respectively. Anti-MBP antibodies were used for detection of the MBP proteins after pulldown. The reactions without E1, E2, or Cul3/Rbx1, and the reactions with MBP-GFP or GST-GFP were used as negative controls. The anti-GST antibody–HRP (sc-459 HRP) was from Santa Cruz Biotechnology (Heidelberg, Germany), the anti-MBP monoclonal antibody (E8032S) was from New England Biolabs (Evry, France) and the streptavidin-HRP conjugate (RPN1231) was from GE-Healthcare (Velizy Villacoublay, France).

### Protoplast transfection and co-immunoprecipitation

*A. thaliana* ecotype Columbia cell suspension cultures were used for protoplast isolation. Isolation and transfection of Arabidopsis protoplasts was performed as described previously (Zhang *et al.*, 2017) using myc-BOP1, myc-BOP2 and haemagglutinin (HA)-LFY constructs.

## Results

### *LFY* and *BOP* genes regulate bract development

During flower development, *BOP* and *LFY* genes have been implicated in the control of some common processes such as the suppression of bracts and *AP1* induction (Norberg *et al.*, 2005; Xu *et al.*, 2010; Siriwardana & Lamb, 2012; Khan *et al.*, 2014). To investigate their genetic interaction in detail, we characterized bract formation on the main inflorescence in various mutant combinations. As shown in Fig. 1, LFY appears to repress bract formation mainly in the lower part of the inflorescence, while BOP1 and BOP2 act mainly in the upper part. However, there is clear evidence that LFY and BOP2 do not simply work independently. Indeed, the *lfy bop2* double mutant triggers > 90% of bract outgrowth along the stem, while single *lfy* and *bop2* mutants display on average 45% and 8% bract outgrowth respectively, with a striking effect on the upper part of the inflorescence that is barely affected in single mutants. In addition, the *lfy* mutation also triggers similar bract outgrowth in the *bop2*/+ background, further supporting that LFY and BOP work together in this process. This genetic interaction is unlikely due to a downregulation of *LFY* gene expression in the *bop2*/+ background as *BOP* genes regulate *LFY* expression only in combination with *APETALA2*-like genes such as PUCHI (Xu *et al.*, 2010; Chandler & Werr, 2017).

We concluded that *LFY* and *BOP*s genetically interact in the regulation of bract development, with *LFY* and *BOP2* playing a major role and acting both together and independently.

### LFY overexpression phenotype is dependent on the *BOP2* gene

We investigated the nature of the LFY–BOP interaction. Such analysis is complicated by the fact *BOP* genes are shown to regulate *LFY* expression at the transcriptional level (Karim *et al.*, 2009; Xu *et al.*, 2010). To minimize this regulation, we studied the influence of *BOP* genes on plants constitutively expressing *LFY*. For this, the *35S::LFY* construct (Weigel & Nilsson, 1995) was introduced in *bop* mutant backgrounds. Compared with wild-type plants (Fig. 2a,g,m), *35S::LFY* plants formed ectopic flowers at the base of rosette leaves, and secondary inflorescences were converted into terminal flowers subtended by a bract (Fig. 2b,h,n) (Weigel & Nilsson, 1995). Similar phenotypes were observed in *35S::LFY bop1-5* plants (Fig. 2c,i,o), indicating that

BOP1 activity is not required for LFY-dependent floral identity determination. By contrast, the *bop2-2* mutation fully suppressed the *35S::LFY* phenotype: *35S::LFY bop2-2* plants were like wild-types with no ectopic flowers nor shoot-to-flower conversions (Fig. 2e,k,q). In addition, this suppression was observed in *35S::LFY bop2-2*/+ plants (Fig. 2d,j,p), indicating that the *bop2-2* allele becomes dominant over the *35S::LFY* transgene. The complete suppression of *35S::LFY* phenotype was also observed in *35S::LFY bop1 bop2* plants, which phenocopied *bop1 bop2* double mutants with ectopic blade growth (Fig. 2f,l,r).

## LFY activity is post-transcriptionally regulated by *BOP2*

In order to understand at which level *LFY* gene activity is affected by *bop* mutations, we first measured *LFY* transcript level in leaves (Fig. 3a). Compared with *35S::LFY* plants, *LFY* transcript levels were similar in leaves of *35S::LFY*-expressing plants in *bop1-5*, *bop2-2* and *bop1-5 bop2-2* mutant backgrounds, indicating that *bop* mutations do not affect *LFY* transcript level when expressed from the *35S* promoter. To determine whether the LFY protein level was altered, we measured the abundance of LFY protein by western blot in seedling leaves.

As expected, the LFY protein was not detected in wild-type plants (Blázquez *et al.*, 1997) but was present in both *35S::LFY* and *35S::LFY bop* plants (Fig. 3b). In the presence of the *bop2* mutation, LFY level was more variable, with a twofold average reduction (Fig. 3c). However, independently of LFY levels being reduced or not, *35S::LFY bop2* or *35S::LFY bop1 bop2* plants never showed the *35S::LFY* phenotype. Furthermore, individual *bop1 bop2* plants transformed with a *35S::LFY-YFP* construct show no reduction in LFY-YFP levels and no LFY overexpression phenotype compared with wild-type plants transformed with the same construct (Fig. S1). These data indicate that the LFY level is partially dependent on *BOP2* whereas LFY activity is fully dependent on *BOP2*, suggesting that BOP2 controls LFY activity mainly through post-translational processes.

This is further supported by the strong enhancement of the *35S::LFY* phenotype in plants overexpressing both LFY and BOP2, as shown in Fig. 4(a–c). In these *35S::LFY 35S::BOP2* plants, termination of inflorescence meristems occurs early in development as in very strong *35S::LFY* lines (Weigel & Nilsson, 1995) because of the conversion to a terminal flower. Also, BOP2 does not appear to modify significantly the LFY level (Fig. 4d), reinforcing the hypothesis that BOP2 rather controls LFY activity.

As previously described, ectopic *LFY* expression in wild-type plants is sufficient to induce ectopic expression of *AP1* (Parcy *et al.*, 1998), a direct target gene of LFY. To assess LFY transcriptional activity in *bop* mutant backgrounds, we analysed *AP1* expression. Ectopic *AP1* expression was observed in the wild-type and *bop1* backgrounds (Fig. 3a), but not in *35S::LFY bop2*, *35S::LFY bop1 bop2* (Fig. 3a) and *35S::LFY-YFP bop1 bop2* (Fig. S1)

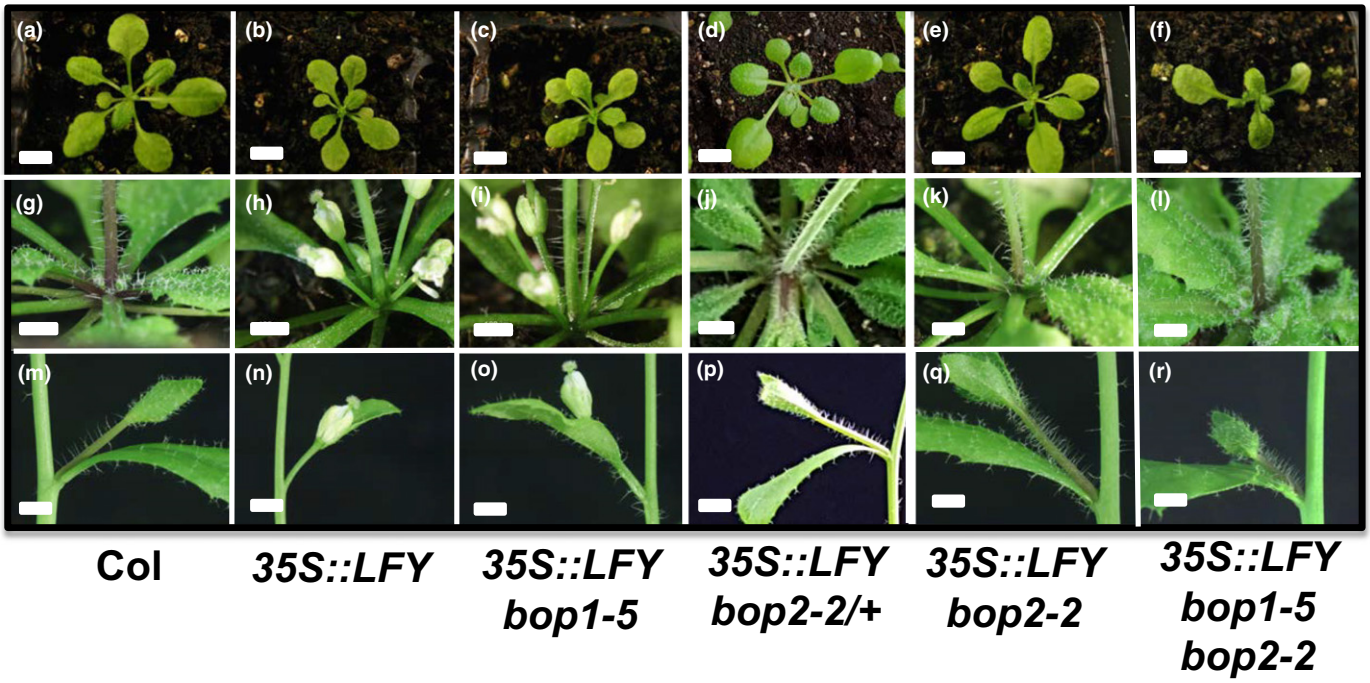


**Fig. 2** The *35S::LFY* phenotype in wild-type and *bop* mutant backgrounds of *Arabidopsis thaliana*. (a–f) Rosettes of plants grown in long days (4 wk). Plants ectopically expressing LFY in a (b) wild-type or (c) *bop1-5* backgrounds grow at a slow rate compared with *bop2* mutant backgrounds (e, f) than wild-type plants (a). Leaf initiation rate of *bop1 bop2* double mutants is also slower (Norberg *et al.*, 2005). (g–l) Ectopic flower formation at rosette leaf axils. Wild-type rosettes show only leaves (g), whereas *35S::LFY* plants develop ectopic flowers at the base of rosette leaves (h, i) except in (j) *bop2*/+ and (k, l) *bop2* mutant backgrounds. Plants were grown for 6 wk in long days. (m–r) Secondary shoots on the main axis. Secondary shoots are formed in the axil of wild-type cauline leaves (m), whereas *35S::LFY* plants develop ectopic flowers subtended by a bract (n, o), except in (p) the *bop2*/+ and (q, r) *bop2* backgrounds that are similar to wild-type. Twenty plants were observed. The phenotypes described are very robust and were seen in all plants of a given genotype. Bars: (a–f) 1 cm; (g–r) 2 mm. BOP, BLADE ON PETIOLE; LFY, LEAFY.

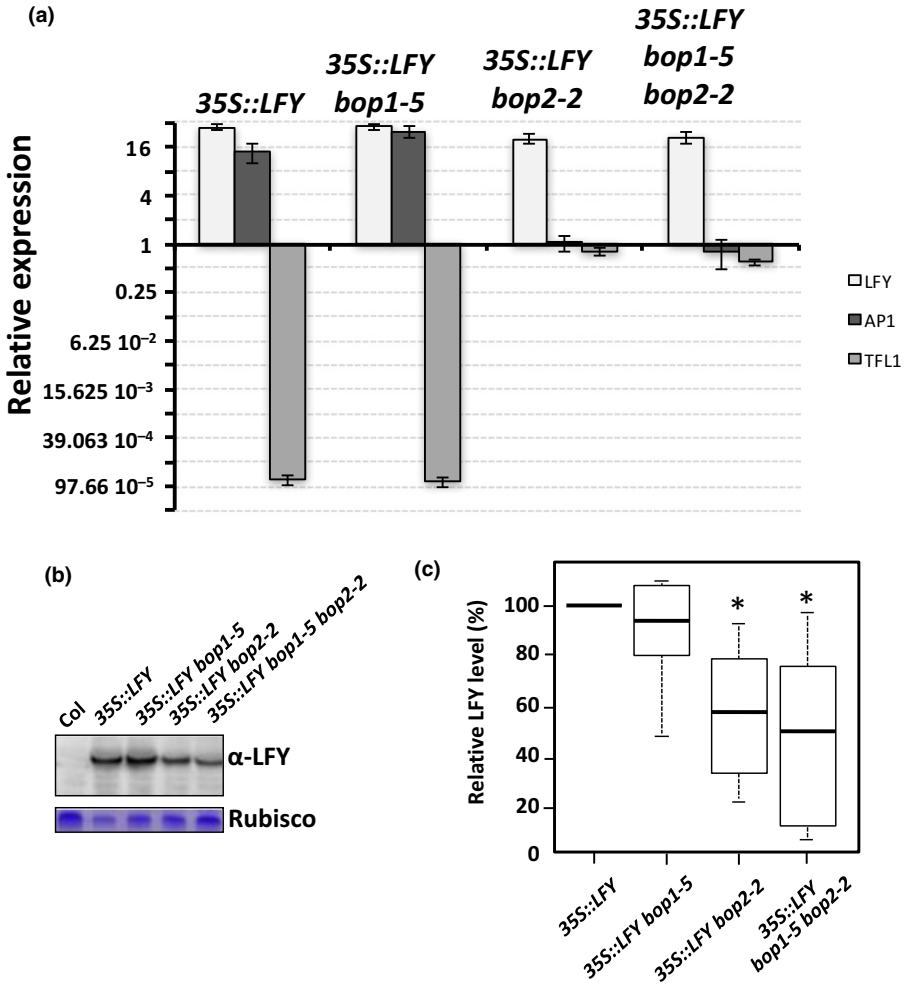


**Fig. 3** LFY expression and activity in wild-type and *bop* mutant backgrounds of *Arabidopsis thaliana*. (a) Relative expression of *LFY*, *AP1* and *TFL1*. *LFY* transcript level is similar in all genotypes, while *AP1* transcript level is strongly reduced and *TFL1* transcript level is strongly de-repressed in *bop2* mutant backgrounds. Error bars represent standard deviation. (b) LFY protein level in wild-type and *bop* mutant backgrounds. While LFY is not detected in wild-type rosette leaves, LFY protein level in *35S::LFY* plants is only mildly affected by *bop1* and *bop2* mutations. Isolated leaves from 16-d-old seedlings grown in long days in Petri dishes were used. (c) Relative LFY protein level in wild-type and *bop* mutant backgrounds. The box plot represents LFY protein level in eight biological replicates, with whiskers indicating minimal and maximum values for each genotype. On average, a twofold reduction is observed when the *bop2* background is present. LFY level in *35S::LFY* plants was used for normalization. Asterisks represent a significant difference according to the Kruskal–Wallis test followed by a post-hoc nonparametric Tukey-type test. *AP1*, *APETALA1*; BOP, BLADE ON PETIOLE; LFY, LEAFY; *TFL1*, *TERMINAL FLOWER1*.

plants where *AP1* expression was completely abolished. We looked at the expression of *TERMINAL FLOWER1* (*TFL1*), a direct target of LFY whose expression is downregulated by AP1 (Ratcliffe *et al.*, 1999; Chahtane *et al.*, 2013). Consistent with the regulation of *AP1* expression, the downregulation of *TFL1* in plants overexpressing LFY was observed in wild-type and *bop1* backgrounds but not in *bop2* or *bop1 bop2* backgrounds (Fig. 3a). This result suggests that LFY transcriptional activity is *BOP2*-dependent. We also analysed whether BOP controls *AP1* expression in response to endogenous *LFY* expression (Fig. 5a,b). We used a short-day to long-day shift protocol where *AP1* induction depends on LFY (Xu *et al.*, 2010; Benlloch *et al.*, 2011). We observed that the *bop1 bop2* double mutation strongly decreases *AP1::GUS* (Fig. 5a) and *AP1* messenger RNA level (Fig. 5b) expression in this time window, without affecting LFY messenger RNA level (Fig. 5b), consistent with the hypothesis that BOP proteins are required for LFY protein to be fully active.

To determine whether LFY DNA-binding activity was controlled by *BOP2*, we next looked at LFY binding to the *AP1* promoter in *bop* mutant background using chromatin immunoprecipitation PCR. As illustrated in Fig. 5(c), the PCR-amplified *AP1* fragment was present in similar amounts in *35S::LFY* and *35S::LFY bop1 bop2* plants. Thus, although *AP1* upregulation by LFY was fully dependent on BOP2, LFY DNA-binding activity does not appear to require BOP factors, suggesting that BOP2 plays a role in the activation of transcription by LFY.

Altogether, these results show that *BOP2* is required for maintaining both LFY activity in leaves and axillary meristems.

## LFY physically interacts with BOP2 protein in plant cells

BOP proteins and their homologue NPR1 have been shown to act as transcriptional co-activators by physically interacting with transcription factors of the TGA family (Després *et al.*, 2000;

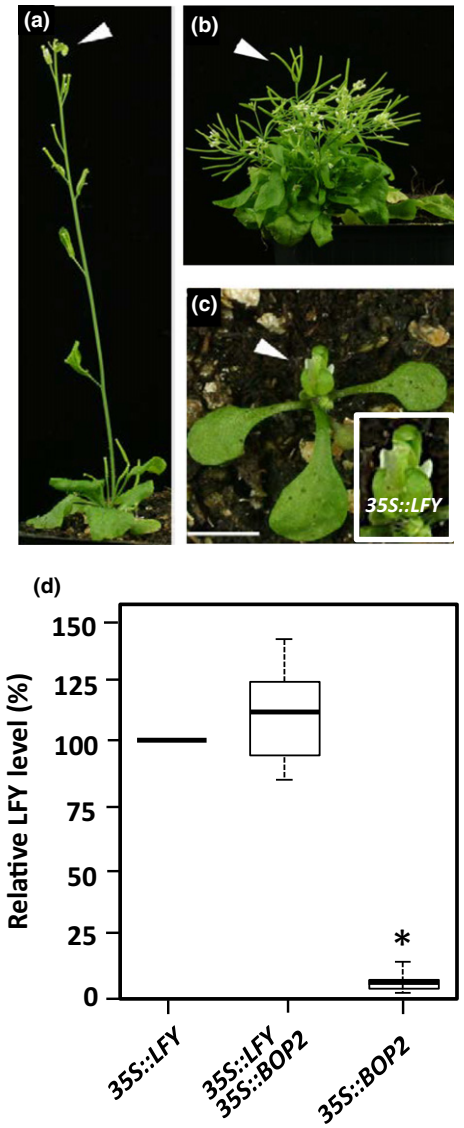


**Fig. 4** Increase of LFY activity by BOP2 in *Arabidopsis thaliana* transgenic plants. (a) A representative *35S::LFY* plant. After formation of a few flowers along the primary stem, a terminal flower is formed leading to stem growth arrest (white arrowhead). (b) A representative *35S::BOP2* plant. Terminal flowers occasionally on stems (white arrowhead). (c) A representative *35S::LFY 35S::BOP2* plant. Plants flower very early, and primary stem growth is stopped after terminal flower formation (white arrowhead). Bar, 1 cm. (d) Relative LFY protein level in wild-type and *35S::BOP2* mutant backgrounds. The box plot represents LFY protein level in three biological replicates, with whiskers indicating minimal and maximum values for each genotype. LFY level in *35S::LFY* plants was used for normalization. The asterisk represents a significant difference according to the Kruskal–Wallis test followed by a post-hoc nonparametric Tukey-type test. Isolated leaves from 4-wk-old seedlings grown in long days on soil were used. BOP, BLADE ON PETIOLE; LFY, LEAFY.

Hepworth *et al.*, 2005; Johnson *et al.*, 2008). Recently, BOP2 was shown to physically interact with the transcription factor PIF4 to regulate its level (Zhang *et al.*, 2017). We therefore wondered whether BOPs and LFY could interact in plant cells. Transient expression of tagged BOP and LFY proteins in protoplasts followed by co-immunoprecipitation showed that HA-tagged LFY was immunoprecipitated together with either myc-BOP1 or myc-BOP2 proteins (Fig. 6a), suggesting that LFY interacts with both BOP1 and BOP2 proteins. To confirm that BOP2 interacts with LFY in plant cells, bimolecular fluorescence complementation assays were carried out between LFY and BOP2 (Fig. 6b). When co-expressing BOP2-CYFP with LFY-NYFP, YFP signal was detected in nuclei of plant cells, as well as in the cytoplasm.

Taken together, these results indicate that LFY establishes a direct interaction with BOP1 and BOP2 proteins both *in vitro* and *in planta*.

## LFY is likely ubiquitinated *in vitro* in a BOP2- and CUL3-dependent manner

Previous studies suggest that overexpressed LFY-FLAG, or tightly associated components, might be ubiquitinated *in vivo* (Chae *et al.*, 2008). Whether LFY can be a direct substrate of ubiquitination complexes has never been addressed *in vitro*. We utilized a GST-LFY fusion protein in standard *in vitro* ubiquitination assays together with biotinylated-ubiquitin, E1, E2 and CRL3-RBX1 and MBP-tagged BOP2 (Zhang *et al.*, 2017). Transfer of biotinylated-ubiquitins onto LFY were monitored by western blot against the streptavidin-HRP (Fig. 7). While the GST-GFP control protein showed no detectable ubiquitinated signals (Fig. 7, lane 6), two main bands were observed when LFY was present (Fig. 7, lane 7).

A first *c.* 115 kDa Ub-containing product was detected when all components of the reaction were present (Fig. 7, lane 7). Omission of either GST-LFY, MBP-BOP2 or CUL3A-RBX1 failed to yield this specific band strongly, suggesting that LFY is a target for ubiquitination. The size of this product is well above the expected GST-LFY size (72 kDa), indicating that LFY might be ubiquitinated.

A second Ub-containing product was also detected in the absence of CUL3A-RBX1 or BOP2 when Ub, E1, E2 and GST-LFY were present (Fig. 7, lanes 4, 5 and 7). Interestingly, this band was not present when GST-PIF4 was used instead of GST-LFY in similar conditions (Zhang *et al.*, 2017), suggesting that it is LFY dependent.

For both bands, it is unclear whether they are ubiquitinated forms of full-length or truncated versions of the GST-LFY fusion (Fig. 7a). Interestingly, the BOP2–LFY interaction seems to be stronger when all components are present, as more BOP2 seems to be co-immunoprecipitated (Fig. 7d, lane 7) compared with reactions with missing components (Fig. 7d, lanes 2, 3 and 4).

## Mutations in *CUL3A/B* genes affect *35S::LFY* in a similar manner as a *bop2* mutation

Several BTB domain proteins, including BOP-like proteins such as NPR3 and NPR4 (Pintard *et al.*, 2004; Fu *et al.*, 2012), are known to participate in E3 ligase complexes involving CUL3 protein. Moreover, a recent study has reported a physical interaction between BOP1 or BOP2 and CUL3A (Zhang *et al.*, 2017), strongly suggesting that BOP and CUL3 act

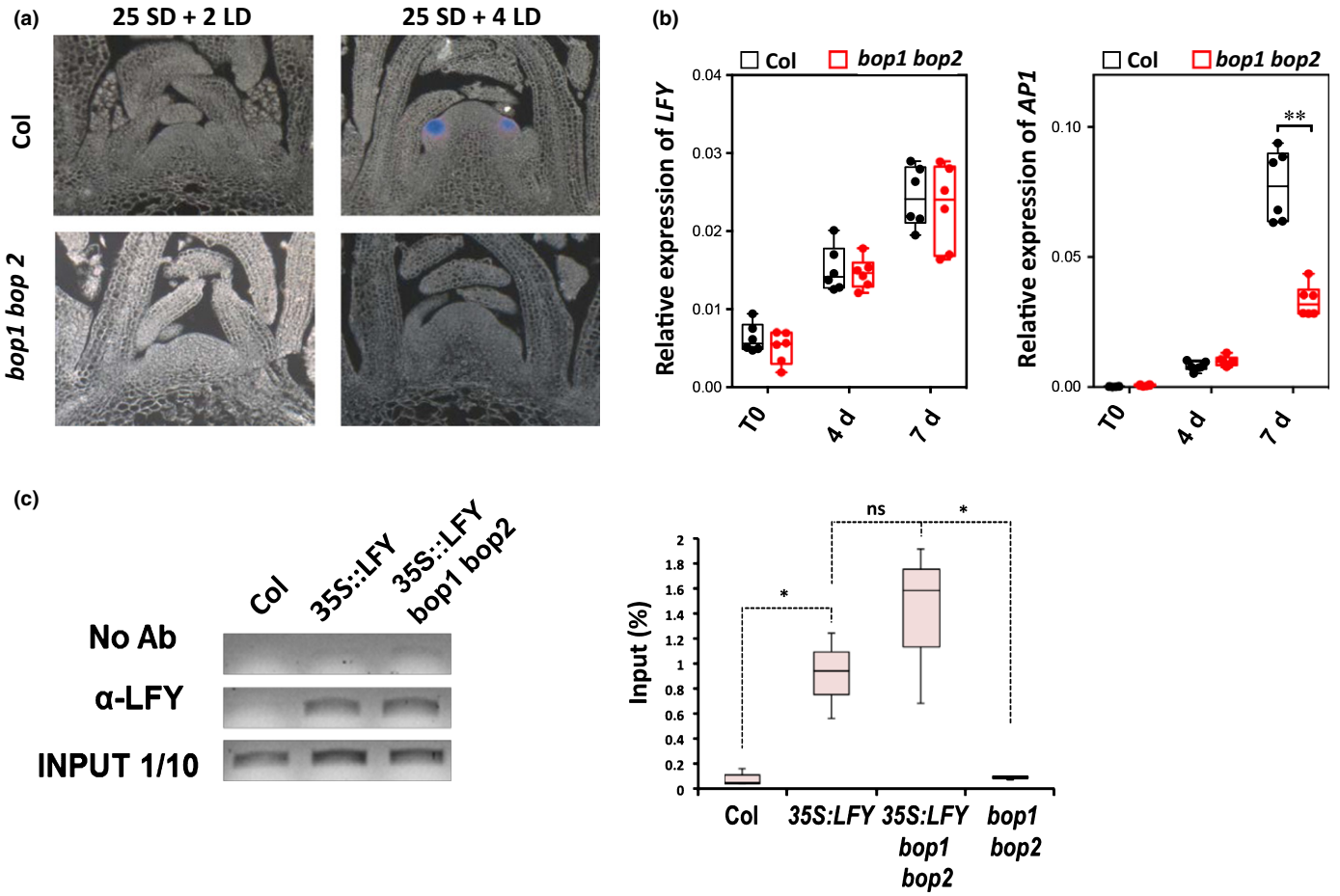


**Fig. 5** BOP-dependent expression of *AP1* and LFY association with *AP1* locus in *Arabidopsis thaliana*. (a) Photoperiodic activation of the *AP1* promoter. Transgenic plants harbouring the 2.2 kb wild-type *AP1* promoter fused to the GUS reporter gene (Benlloch *et al.*, 2011) were grown in short day (SD) conditions for 25 d before being shifted to long day (LD) conditions. The activation of the promoter was monitored by localization of the GUS activity in the apex at 2 or 4 d after the shift as indicated. (b) Relative expression levels of *LFY* and *AP1* in wild-type and *bop1 bop2* plants. The box plots represent *LFY* and *AP1* transcript levels in apices of 4-wk-old plants grown in SD for 4 wk then shifted to LD. Apices were harvested before the shift (T0), and 4 and 7 d after shift. Apices from three plants were pooled as one biological replicate. Six biological replicates were analysed by quantitative PCR analysis. The results are shown as box-and-whiskers plots, with circles representing each replicate. Asterisks indicate statistically significant differences between *bop1 bop2* and wild-type plants, tested by Welch-corrected *t*-test (two-tailed): **, $P < 0.01$. (c) A representative semi-quantitative PCR gel showing that LFY associates with *AP1* locus similarly in wild-type and *bop1 bop2* backgrounds. On the right, quantification of LFY association with *AP1* locus. The box plot represents LFY binding in three biological replicates. Asterisks represent a significant difference according to the Kruskal–Wallis test followed by a post-hoc nonparametric Tukey-type test. *, $P < 0.05$; ns, nonsignificant. *AP1*, *APETALA1*; BOP, BLADE ON PETIOLE; LFY, LEAFY.

together in CRL3 complexes. To determine whether CRL3 complexes play a role in LFY activity, we studied the activity of *35S::LFY* in *cul3* mutants. The two *CUL3* genes of Arabidopsis, *CUL3A* and *CUL3B*, are essential for embryo development (Figueroa *et al.*, 2005). We took advantage of the *cul3a-3 cul3b-1* double mutant (Thomann *et al.*, 2005), which is knocked out for *CUL3B* and partially functional for *CUL3A*, to investigate whether LFY is active in a *CUL3*-deficient background. In the $F_1$ generation, all *35S::LFY cul3a-3*/+ *cul3b-1*/+ plants displayed a wild-type phenotype, suggesting that the overexpressed LFY was not functional. Analysis of subsequent generations showed that the weak *cul3a-3* allele was acting as a semi-dominant allele reducing the phenotype of *35S::LFY* (Fig. S2), while the *cul3b-1* allele was acting as a dominant allele (Fig. 8a) fully suppressing the *35S::LFY* phenotype.

The transcriptional activation of *AP1* by *35S::LFY* in leaves was completely abolished in *cul3a* or *cul3b*/+ backgrounds (Fig. 8b) compared with *35S::LFY* plants. Ectopic LFY protein was present in lower amounts in the *cul3a* homozygous background compared with the wild-type background and in amounts comparable to the *bop1 bop2* background (Fig. 8c). In the *cul3b*/+ background, the level of LFY was decreased further. Thus, as for the *BOP2* gene, *CUL3A* and *-B* genes play a role in controlling both LFY levels and LFY activity.

Taken together, these results strongly support that *BOP* genes act together with *CUL3*s in CRL3 complexes and positively control LFY activity in plants. This genetic interaction is reinforced by the phenotype comparisons of *cul3a cul3b* and *bop1 bop2* double mutants. As previously reported (McKim *et al.*, 2008), plants carrying *bop1 bop2* mutations were affected in flower

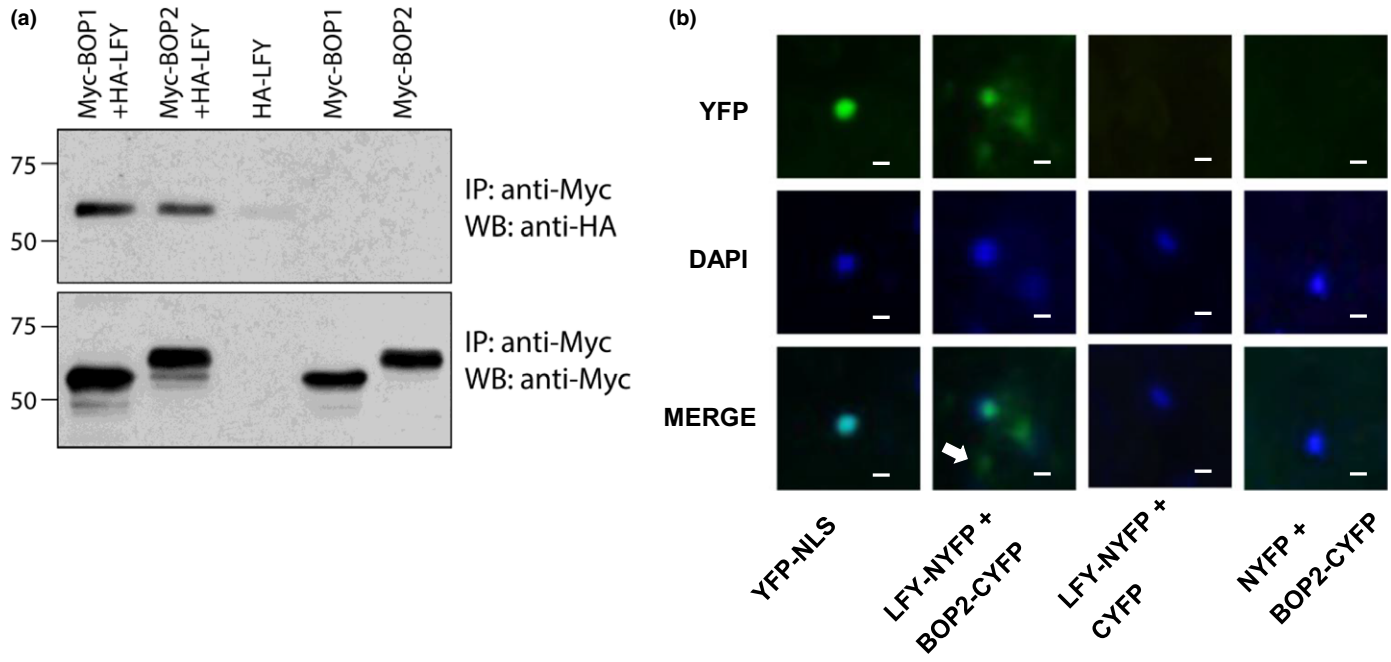


**Fig. 6** Physical interaction between LFY and BOP proteins. (a) Co-immunoprecipitation of LFY and BOP in *Arabidopsis thaliana* protoplasts. Co-immunoprecipitated HA-LFY was detected by western blotting using anti-HA antibodies, after immunoprecipitation of myc-BOP1 or myc-BOP2 by anti-myc antibodies. Precipitate probed with anti-myc antibodies and supernatant probed with anti-HA antibodies are shown as controls. (b) Bimolecular fluorescence complementation assays of LFY and BOP2 interaction in *A. thaliana*. NYFP, N-terminal part of YFP; CYFP, C-terminal part of YFP; YFP–NLS, YFP fused to a nuclear localization signal; BOP, BLADE ON PETIOLE; HA, haemagglutinin; LFY, LEAF. Bars, 10 μm.

development, resulting in a loss of floral organ abscission and delay in senescence, resulting in siliques with permanently attached floral organs compared with the wild-type. Interestingly, *cul3a cul3b* plants also showed defects in floral organ abscission and senescence (Fig. S3) compared with the wild-type. In *cul3a cul3b* plants, floral organs could be detached upon mechanical stress, suggesting that floral organ abscission was partial compared with the *bop1 bop2* phenotype. Thus, these results illustrate that both BOP1/2 and CUL3A/B proteins can regulate a common mechanism, further supporting that BOPs act in CRL3 complexes through CUL3 interaction.

## Discussion

Proteasome-mediated protein degradation plays a pivotal role in many plant growth and developmental processes, including photomorphogenesis and plant hormone signalling (Vierstra, 2009). Transcription factors and transcription co-activators are among targets of the proteasome in several of these processes. In most cases, the proteasome either degrades activators to suppress transcription or degrades repressor proteins to activate gene expression. However, several studies indicate that proteasome-mediated turnover of activators may also be essential for their ability to activate target gene transcription (Collins & Tansey, 2006), such as the immunity master regulator NPR1 (Spoel *et al.*, 2009) in plants whose degradation is necessary for its turnover to promote efficient expression of downstream defence response genes.

LFY is also a major regulator whose activity is regulated by the proteasome. The F-box protein UFO is required together with LFY for the activation of *AP3* expression in developing petal and stamen primordia (Ingram *et al.*, 1995; Jack *et al.*, 1992; Lee *et al.*, 1997; Levin and Meyerowitz, 1995; Wilkinson & Haughn, 1995) and for ectopic activation of *AP3* (Parcy *et al.*, 1998) indicating that a CRL1-based mechanism is involved in LFY activity. Additional studies suggest a regulation of LFY activity via protein degradation and concomitant activation of LFY as well as ubiquitination with UFO functioning as a DNA-associated transcriptional co-factor (Chae *et al.*, 2008).

We present here several lines of evidence that LFY is also post-transcriptionally regulated by CRL3 complexes with BOP2 acting as a substrate adaptor targeting LFY.

### BOP2 and CUL3 proteins are required for both LFY stability and LFY activity

*BOP* and *LFY* genes are involved in similar processes suppressing bract formation and reducing flowering time in short days (Norberg *et al.*, 2005). The work presented here provides evidence that BOP2, CUL3A and -B, all components of CRL3 complexes, are required for the control of LFY protein level and activity in plants.

Interestingly, in the *lfy* and *35S::LFY* backgrounds, the *BOP2* mutant allele acts as a dominant allele, whereas *BOP1* seems to play a minor role. This was also recently shown in the regulation

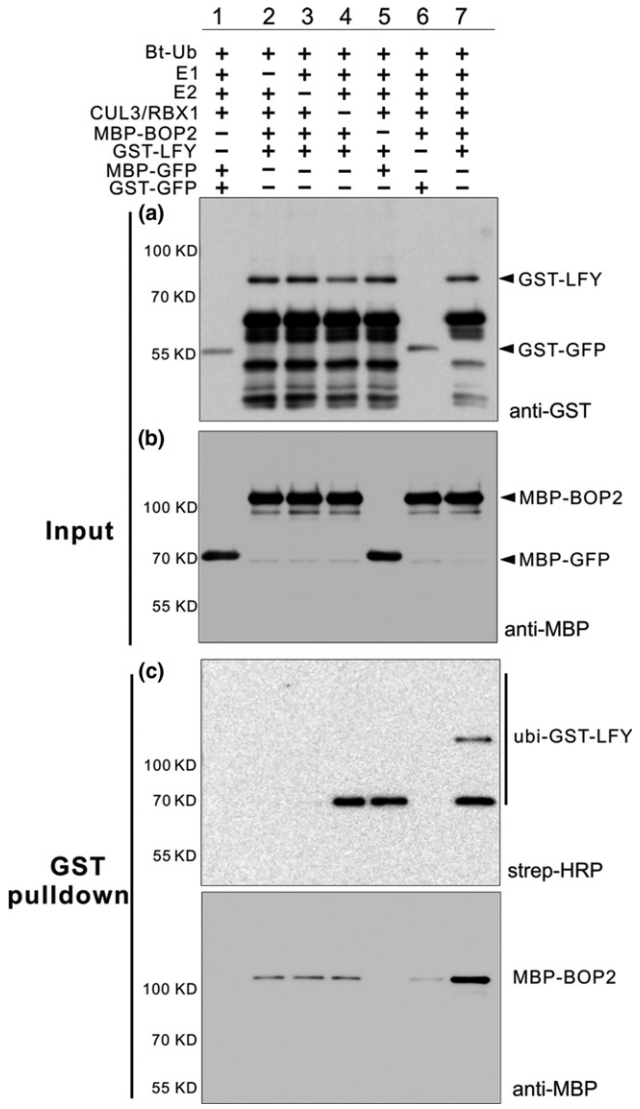


**Fig. 7** CUL3–BOP2 complex mediates the polyubiquitination of LFY in ubiquitination assays. A cullin3 E3 ubiquitin ligase complex was assembled with recombinant MBP–BOP2 and incubated with GST–LFY. Inputs are shown using (a) anti-GST and (b) anti-MBP antibodies. The reactions were then pulled down with glutathione Sepharose 4B beads followed by western blotting analysis using (c) streptavidin–HRP and (d) anti-MBP antibodies. Streptavidin-HRP were used for detection of biotin-labelled ubiquitinated protein. MBP–GFP and GST–GFP were used as negative controls. The human E1, E2 and CUL3/RBX proteins were used. The two main ubiquitinated bands seen in (c) were always observed in our experiments. Some intermediate forms were sometimes observed between these two main bands, as illustrated in Supporting Information Fig. S4. A representative experiment is shown among the three experiments performed. BOP, BLADE ON PETIOLE; CUL3, CULLIN3; GFP, green fluorescent protein; GST, glutathione *S*-transferase; HRP, horseradish peroxidase; LFY, LEAFY; MBP, myelin basic protein; RBX, Ring-Box.

of PIF4 activity, where BOP2 promotes degradation of PIF4 in response to light (Zhang *et al.*, 2017). This suggests that the level of BOP2 protein is critical compared with the BOP1 level or that BOP2 displays specific properties, such as its ability to make stronger interactions with itself (Hepworth *et al.*, 2005) or with CUL3 proteins (Zhang *et al.*, 2017). Regarding *CUL3* genes, both *CUL3A* and *CUL3B* mutant alleles act as dominant alleles in the *35S::LFY* background, even though the CUL3A protein is largely predominantly expressed with respect to CUL3B protein in seedlings (Figueroa *et al.*, 2005).

Although the decrease in LFY level does not account for the complete lack of LFY activity in *bop2* and *cul3* backgrounds, our data show that *BOP2* and *CUL3* genes play a role in maintaining LFY level in seedlings. It is not known how the LFY level is regulated, nor whether it correlates with its DNA binding or transcriptional activities. One hypothesis is that BOP2 binds the fraction of LFY that is not bound to DNA as NPR1 does to TGA2 (Johnson *et al.*, 2008) and that LFY regulation by BOP2 does not fit a simple model in which simply activating transcription is the signal for its degradation and turnover (Muratani & Tansey, 2003). This is in contrast with UFO, which regulates LFY activity but does not affect the overall LFY level, raising the possibility that UFO specifically acts to modulate the stability of promoter-bound LFY (Chae *et al.*, 2008). Thus, our work suggests that CRL3 and CRL1 components have different roles in controlling LFY activity.

Our findings support a fundamental function of CRL3 complexes in the control of a subset of LFY activities in *A. thaliana*. While this control can take place in plants overexpressing LFY in both vegetative tissues, as seen with the regulation of *AP1* and *TFL1* expression, and in axillary meristems, as seen with the control of ectopic flower formation, it remains to be determined whether this regulation occurs in wild-type plant development. The fact that *bop1 bop2* plants do not show an *lfy* phenotype suggests that BOP proteins control only a subset of LFY functions and that additional factors (such as UFO and other unknown factors) are likely important for other LFY activities in the wild-type.

Several observations indicate that *LFY*, *BOP2* and *CUL3A/B* genes participate in the same developmental pathways. Phenotypic similarities in floral development are observed between *lfy* and *bop1 bop2* plants, such as the lack of bract repression or the impaired *AP1* activation, and between *bop1 bop2* and *cul3a cul3b* plants, such as delayed floral organ abscission. In addition, both *lfy* (Winter *et al.*, 2011) and *cul3a cul3b* (Spoel *et al.*, 2009) mutants play a role in the control of pathogen responses. Finally, expression profiles of *LFY*, *BOP2* and *CUL3A/B* genes highly overlap, especially during early floral meristem development (Sessions *et al.*, 2000; Norberg *et al.*, 2005; Thomann *et al.*, 2005). It is possible that additional BTB proteins of Arabidopsis are involved in LFY regulation in floral meristems. Introduction of other *BTB* gene mutations in *bop1 bop2* or the use of conditional *cul3* mutants might be necessary to study this regulation of LFY activity *in planta*.

## LFY is ubiquitinated in a cell-free assay *in vitro*

We show here that LFY physically interacts with BOP1 and BOP2 in plant cells and *in vitro*. As BOP2 interacts with CUL3 proteins (Zhang *et al.*, 2017), it is tempting to speculate that LFY is a substrate of a CRL3 complex with BOP2 acting as an adaptor between CUL3A and LFY. In a reconstituted *in vitro*

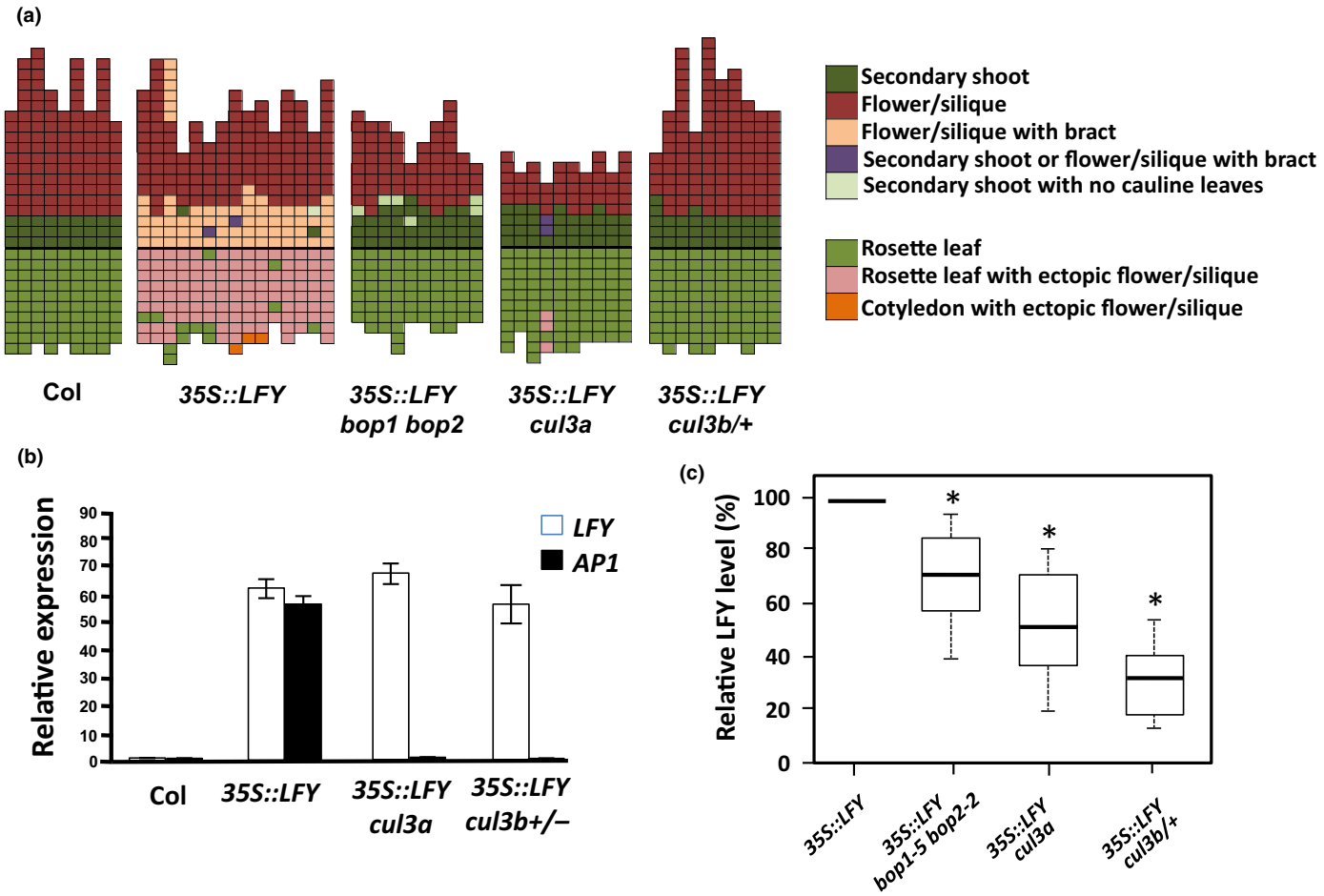


**Fig. 8** *35S::LFY* phenotype in *Arabidopsis thaliana cul3* mutant backgrounds. (a) Phenotype analysis of *35S::LFY* in wild-type and *cul3* backgrounds. Each column represents a single plant from bottom to top, with each square representing a phenotypic observation. The thick horizontal black line represents the rosette–stem junction. Note that *35S::LFY cul3b−/−* plants could not be obtained possibly because the *35S::LFY* transgene is genetically linked to the *CUL3B* locus. Plants were grown for 6 wk. (b) *LFY* and *AP1* transcript levels in *cul3* backgrounds. Error bars represent SE. (c) Relative LFY protein level in rosette leaves of *cul3* mutant backgrounds. The box plot represents LFY protein level in three biological replicates, with whiskers indicating minimal and maximum values for each genotype. LFY level in *35S::LFY* plants was used for normalization. Asterisks represent a significant difference according to the Kruskal–Wallis test followed by a post-hoc nonparametric Tukey-type test. Mutations of *bop* or *cul3* affect LFY proteins level in a similar manner. *AP1*, *APETALA1*; CUL3B, CULLIN3B; LFY, LEAFY.

ubiquitination assay, we show that incubation of GST-LFY, but not GST-GFP, together with all other standard components, produced a specific *c.* 115 kDa Ub-containing product. Omission of either LFY, BOP2 or CUL3A failed to yield this specific band, strongly suggesting that LFY is a target for ubiquitination.

Interestingly, a second Ub-containing product was also detected in the absence of CUL3A or BOP2 when Ub, E1, E2 and LFY were present. This product shows a molecular weight that is compatible with monoubiquitination of LFY. Although we cannot exclude a direct ubiquitination by the human E2 used as reported previously (David *et al.*, 2010), the ability of obtain an LFY-dependent ubiquitinated band might indicate that LFY possesses an intrinsic E3 ligase activity even if the examination of its sequence does not reveal any obvious domain with such an activity. Transcription factors have been reported to display an E3 ligase activity, such as the human hDREF (Yamashita *et al.*, 2016), or viral transcription factors, which also display an auto-ubiquitination activity (Yu *et al.*, 2005). These two putative forms of LFY ubiquitination are reminiscent of the regulation of FOXO transcription factor activity in animals with a monoubiquitinated form displaying increased transcriptional activity, whereas polyubiquitinated forms are degraded by the proteasome (van der Horst *et al.*, 2006). It remains to be determined whether several lysines of LFY are monoubiquitinated or whether a single lysine is polyubiquitinated or alternatively whether a combination of both mono- and polyubiquitinations are involved. Furthermore, identification of LFY lysine residues targeted for ubiquitination in the two evolutionarily conserved domains of LFY (Sayou *et al.*, 2014, 2016) will allow one to determine whether LFY ubiquitination is a conserved mechanism of LFY regulation.

Combined with previous results (Xu *et al.*, 2010), our findings indicate that BOP might act both as a transcriptional co-activator (recruited by PERIANTHIA in an LFY-independent manner) and as an adaptor for cullin, just as NPR3 and NPR4, which are both adaptors between CUL3 and NPR1 and co-activators of

TGA transcription factors (Fu *et al.*, 2012). These two functions might actually be linked, and recruitment of BOP on promoters where LFY is also bound might be the key for synergistic gene activation by both proteins.

### Dual regulation of LFY activity by SCF and CRL3 complexes

Our data indicate that LFY is likely regulated by ubiquitination by CRL3 complexes with BOP2 acting as an adaptor bridging CUL3A/B with LFY. This suggests a model in which flower development is controlled via protein degradation and concomitant activation of LFY. As for other proteins (van der Horst *et al.*, 2006; Spoel *et al.*, 2009), LFY would be targeted by a $CRL3^{BOP2}$ complex and ubiquitinated for protein degradation through the 26S proteasome system after interacting with the transcriptional machinery, thus allowing new LFY molecules to bind on *cis*-regulatory regions. This turnover would concern a small portion of LFY proteins, as BOP2 does not seem to regulate the overall DNA-bound LFY fraction, as seen with chromatin immunoprecipitation PCR on the *AP1* locus.

Our work reveals an intriguing aspect of LFY, which is its dual regulation by two distinct E3 ubiquitin ligases. To our knowledge, only one transcription factor is known to be regulated by such a dual CRL1 and CRL3 E3 ligase targeting. In *Drosophila*, Cubitus interruptus (Ci) is a zinc-finger transcription factor that plays a role in the signal transduction cascade of the morphogen peptide Hedgehog (Hh) (Ou *et al.*, 2002; Zhang *et al.*, 2006). In the absence of Hh, Ci is targeted to the $SCF^{Slimb}$-based E3 ligase complex that mediates its partial degradation, which leaves the DNA binding domain acting as a repressor of target genes; by contrast, at high Hh levels, a $CUL3^{BTB}$-based E3 ligase forms a negative feedback loop to target full-length Ci for complete degradation. Intermediate levels of Hh define a boundary between the anterior and posterior parts of organ primordia where Ci activates Hh response genes. Interestingly, both UFO and BOP help to define boundary regions in the floral meristem or leaves (Durfee *et al.*, 2003; Žádníková & Simon, 2014), and it would be of interest to determine whether LFY could participate in the establishment of boundaries in the floral meristem by a dual post-transcriptional regulation. Nevertheless, LFY seems to be unique, since both $SCF^{UFO}$ and $CUL3^{BOP}$ E3 ligases are required for its activity rather than inactivating it.

## Acknowledgements

We thank Pascal Genschik for the gift of the *cul3a cul3b* double mutant. Our work was supported by the Agence Nationale de la Recherche projects (ANR-10-LABX-49-01 and ANR-16-CE92-0023), a PhD Université Grenoble Alpes fellowship to H.C. and The Kempe Foundation fellowship to R.B.

## Author contributions

O.N., F.P. and G.V. planned and designed the research. H.C., M.L.M. and M.N. performed the genetic and *in planta* experiments and analysed the data. E.T. performed the molecular biology experiments. B.Z. performed the *in vitro* ubiquitination assays. L.B. performed the immunoprecipitation from protoplasts. R.B. performed experiments with AP1:GUS transgenic plants. M.H. performed the reverse transcription quantitative PCR experiments. G.V. performed the genetic experiments and wrote the paper with the help of F.P. and input from all authors.

## ORCID

Hicham Chahtane http://orcid.org/0000-0002-0976-1186

## References

**Azimzadeh J, Nacry P, Christodoulidou A, Drevensek S, Camilleri C, Amiour N, Parcy F, Pastuglia M, Bouchez D. 2008.** *Arabidopsis* TONNEAU1 proteins are essential for preprophase band formation and interact with centrin. *Plant Cell* **20**: 2146–2159.

**Benlloch R, Kim MC, Sayou C, Thévenon E, Parcy F, Nilsson O. 2011.** Integrating long-day flowering signals: a LEAFY binding site is essential for proper photoperiodic activation of *APETALA1*. *Plant Journal* **67**: 1094–1102.

**Blázquez M, Soowal LN, Lee I, Weigel D. 1997.** *LEAFY* expression and flower initiation in *Arabidopsis*. *Development* **124**: 3835–3844.

**Chae E, Tan QK, Hill TA, Irish VF. 2008.** An *Arabidopsis* F-box protein acts as a transcriptional co-factor to regulate floral development. *Development* **1245**: 1235–1245.

**Chahtane H, Vachon G, Le Masson M, Thévenon E, Périgon S, Mihajlovic N, Kalinina A, Michard R, Moyroud E, Monniaux M *et al.* 2013.** A variant of LEAFY reveals its capacity to stimulate meristem development by inducing *RAX1*. *Plant Journal* **74**: 678–689.

**Chandler J, Werr W. 2017.** DORNRÖSCHEN, DORNRÖSCHEN-LIKE, and PUCHI redundantly control floral meristem identity and organ initiation in Arabidopsis. *Journal of Experimental Botany* **68**: 3457–3472.

**Choi CM, Gray WM, Mooney S, Hellmann H. 2014.** Composition, roles, and regulation of cullin-based ubiquitin E3 ligases. *Arabidopsis Book* **12**: 1–36.

**Collins GA, Tansey WP. 2006.** The proteasome: a utility tool for transcription? *Current Opinion in Genetics & Development* **16**: 197–202.

**Czechowski T, Bari RP, Stitt M, Scheible WR, Udvardi MK. 2004.** Real-time RT-PCR profiling of over 1400 *Arabidopsis* transcription factors: unprecedented sensitivity reveals novel root- and shoot-specific genes. *Plant Journal* **38**: 366–379.

**David Y, Ziv T, Admon A, Navon A. 2010.** The E2 ubiquitin-conjugating enzymes direct polyubiquitination to preferred lysines. *Journal of Biological Chemistry* **285**: 8595–8604.

**Denay G, Chahtane H, Tichtinsky G, Parcy F. 2017.** A flower is born: an update on Arabidopsis floral meristem formation. *Current Opinion in Plant Biology* **35**: 15–22.

**Després C, DeLong C, Glaze S, Liu E, Fobert PR. 2000.** The Arabidopsis NPR1/NIM1 protein enhances the DNA binding activity of a subgroup of the TGA family of bZIP transcription factors. *Plant Cell* **12**: 279–290.

**Duda DM, Borg LA, Scott DC, Hunt HW, Hammel M, Schulman BA. 2008.** Structural insights into NEDD8 activation of Cullin-RING ligases: conformational control of conjugation. *Cell* **134**: 995–1006.

**Dumas R, Joyard J, Douce R. 1989.** Purification and characterization of acetohydroxyacid reductoisomerase from spinach chloroplasts. *Biochemical Journal* **262**: 971–976.

**Durfee T, Roe JL, Sessions RA, Inouye C, Serikawa K, Feldmann KA, Weigel D, Zambryski PC. 2003.** The F-box-containing protein UFO and AGAMOUS participate in antagonistic pathways governing early petal development in Arabidopsis. *Proceedings of the National Academy of Sciences, USA* **100**: 8571–8576.

**Figueroa P, Gusmaroli G, Serino G, Habashi J, Ma L, Shen Y, Feng S, Bostick M, Callis J, Hellmann H *et al.* 2005.** Arabidopsis has two redundant Cullin3 proteins that are essential for embryo development and that interact with RBX1

and BTB proteins to form multisubunit E3 ubiquitin ligase complexes *in vivo*. *Plant Cell* **17**: 1180–1195.

**Fu ZQ, Yan S, Saleh A, Wang W, Ruble J, Oka N, Mohan R, Spoel SH, Tada Y, Zheng N *et al.* 2012.** NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. *Nature* **486**: 228–232.

**Gagne JM, Downes BP, Shiu S-H, Durski AM, Vierstra RD. 2002.** The F-box subunit of the SCF E3 complex is encoded by a diverse superfamily of genes in Arabidopsis. *Proceedings of the National Academy of Sciences, USA* **99**: 11519–11524.

**Gehl C, Waadt R, Mendel R, Ha R. 2009.** New GATEWAY vectors for high throughput analyses of protein–protein interactions by bimolecular fluorescence complementation. *Molecular Plant* **2**: 1051–1058.

**Guénin S, Mauriat M, Pelloux J, Van Wuytswinkel O, Bellini C, Gutierrez L. 2009.** Normalization of qRT-PCR data: the necessity of adopting a systematic, experimental conditions-specific, validation of references. *Journal of Experimental Botany* **60**: 487–493.

**Hepworth SR, Klenz JE, Haughn GW. 2006.** UFO in the *Arabidopsis* inflorescence apex is required for floral-meristem identity and bract suppression. *Planta* **223**: 769–778.

**Hepworth SR, Zhang Y, McKim S, Li X, Haughn GW. 2005.** BLADE-ON-PETIOLE-dependent signaling controls leaf and floral patterning in Arabidopsis. *Plant Cell* **17**: 1434–1448.

**van der Horst A, de Vries-Smits AMM, Brenkman AB, van Triest MH, van den Broek N, Colland F, Maurice MM, Burgering BMT. 2006.** FOXO4 transcriptional activity is regulated by monoubiquitination and USP7/HAUSP. *Nature Cell Biology* **8**: 1064–1073.

**Ingram GC, Goodrich J, Wilkinson MD, Simon R, Haughn GW, Coen ES. 1995.** Parallels between UNUSUAL FLORAL ORGANS and FIMBRIATA, genes controlling flower development in Arabidopsis and Antirrhinum. *The Plant Cell* **7**: 1501–1510.

**Jack T, Brockman LL, Meyerowitz EM. 1992.** The homeotic gene APETALA3 of *Arabidopsis thaliana* encodes a MADS box and is expressed in petals and stamens. *Cell* **68**: 683–697.

**Johnson C, Mhatre A, Arias J. 2008.** NPR1 preferentially binds to the DNA-inactive form of *Arabidopsis* TGA2. *Biochimica et Biophysica Acta – Gene Regulatory Mechanisms* **1779**: 583–589.

**Jun JH, Ha CM, Fletcher JC. 2010.** BLADE-ON-PETIOLE1 coordinates organ determinacy and axial polarity in *Arabidopsis* by directly activating ASYMMETRIC LEAVES2. *Plant Cell* **22**: 62–76.

**Karim MR, Hirota A, Kwiatkowska D, Tasaka M, Aida M. 2009.** A role for *Arabidopsis PUCHI* in floral meristem identity and bract suppression. *Plant Cell* **21**: 1360–1372.

**Khan M, Xu H, Hepworth SR. 2014.** *BLADE-ON-PETIOLE* genes: setting boundaries in development and defense. *Plant Science* **215–216**: 157–171.

**Kwak J, Workman JL, Lee D. 2011.** The proteasome and its regulatory roles in gene expression. *Biochimica et Biophysica Acta* **1809**: 88–96.

**Lee I, Wolfe DS, Nilsson O, Weigel D. 1997.** A LEAFY co-regulator encoded by UNUSUAL FLORAL ORGANS. *Current Biology* **7**: 95–104.

**Levin JZ, Meyerowitz EM. 1995.** UFO: an Arabidopsis gene involved in both floral meristem and floral organ development. *The Plant Cell* **7**: 529–548.

**Lenhard M, Bohnert A, Jürgens G, Laux T. 2001.** Termination of stem cell maintenance in *Arabidopsis* floral meristems by interactions between *WUSCHEL* and *AGAMOUS*. *Cell* **105**: 805–814.

**Li W, Zhou Y, Liu X, Yu P, Cohen JD, Meyerowitz EM. 2013.** LEAFY controls auxin response pathways in floral primordium formation. *Science Signaling* **6**: ra23.

**Liu C, Xi W, Shen L, Tan C, Yu H. 2009.** Regulation of floral patterning by flowering time genes. *Developmental Cell* **16**: 711–722.

**Lohmann JU, Hong RL, Hobe M, Busch MA, Parcy F, Simon R, Weigel D. 2001.** A molecular link between stem cell regulation and floral patterning in *Arabidopsis*. *Cell* **105**: 793–803.

**Maizel A, Weigel D. 2004.** Temporally and spatially controlled induction of gene expression in *Arabidopsis thaliana*. *Plant Journal* **38**: 164–171.

**McKim SM, Stenvik G-E, Butenko MA, Kristiansen W, Cho SK, Hepworth SR, Aalen RB, Haughn GW. 2008.** The *BLADE-ON-PETIOLE* genes are essential for abscission zone formation in *Arabidopsis*. *Development* **135**: 1537–1546.

**Moyroud E, Kusters E, Monniaux M, Koes R, Parcy F. 2010.** LEAFY blossoms. *Trends in Plant Science* **15**: 346–352.

**Moyroud E, Minguet EG, Ott F, Yant L, Posé D, Monniaux M, Blanchet S, Bastien O, Thévenon E, Weigel D *et al.* 2011.** Prediction of regulatory interactions from genome sequences using a biophysical model for the *Arabidopsis* LEAFY transcription factor. *Plant Cell* **23**: 1293–1306.

**Muratani M, Tansey WP. 2003.** How the ubiquitin–proteasome system controls transcription. *Nature Reviews Molecular Cell Biology* **4**: 192–201.

**Ni W, Xu S, Tepperman JM, Stanley DJ, Maltby DA, Gross JD, Burlingame AL, Wang Z, Quail PH. 2015.** A mutually assured destruction mechanism attenuates light signaling in *Arabidopsis*. *Science* **344**: 1160–1164.

**Nilsson O, Lee I, Blázquez MA, Weigel D. 1998.** Flowering-time genes modulate the response to *LEAFY* activity. *Genetics* **150**: 403–410.

**Norberg M, Holmlund M, Nilsson O. 2005.** The *BLADE ON PETIOLE* genes act redundantly to control the growth and development of lateral organs. *Development* **132**: 2203–2213.

**Ou C-Y, Lin Y-F, Chen Y-J, Chien C-T. 2002.** Distinct protein degradation mechanisms mediated by Cul1 and Cul3 controlling Ci stability in *Drosophila* eye development. *Genes & Development* **16**: 2403–2414.

**Parcy F. 2005.** Flowering: a time for integration. *International Journal of Developmental Biology* **49**: 585–593.

**Parcy F, Nilsson O, Busch MA, Lee I, Weigel D. 1998.** A genetic framework for floral patterning. *Nature* **395**: 561–566.

**Pfaffl MW. 2001.** A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* **29**: 16–21.

**Pfaffl MW, Horgan GW, Dempfle L. 2002.** Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Research* **30**: e36.

**Pintard L, Willems A, Peter M. 2004.** Cullin-based ubiquitin ligases: Cul3–BTB complexes join the family. *EMBO Journal* **23**: 1681–1687.

**Ratcliffe OJ, Bradley DJ, Coen ES. 1999.** Separation of shoot and floral identity in Arabidopsis. *Development* **126**: 1109–1120.

**Risseeuw E, Venglat P, Xiang D, Komendant K, Daskalchuk T, Babic V, Crosby W, Datla R. 2013.** An activated form of UFO alters leaf development and produces ectopic floral and inflorescence meristems. *PLoS ONE* **8**: e83807.

**Saddic L, Huvermann B, Bezhani S, Su Y, Winter CM, Kwon CS, Collum RP, Wagner D. 2006.** The LEAFY target LMI1 is a meristem identity regulator and acts together with LEAFY to regulate expression of *CAULIFLOWER*. *Development* **133**: 1673–1682.

**Sawa S, Watanabe K, Goto K, Kanaya E, Morita EH, Okada K. 1999.** *FILAMENTOUS FLOWER*, a meristem and organ identity gene of *Arabidopsis*, encodes a protein with a zinc finger and HMG-related domains. *Genes and Development* **13**: 1079–1088.

**Sayou C, Monniaux M, Nanao MH, Moyroud E, Brockington SF, Thévenon E, Chahtane H, Warthmann N, Melkonian M, Zhang Y *et al.* 2014.** A promiscuous intermediate underlies the evolution of LEAFY DNA binding specificity. *Science* **343**: 645–648.

**Sayou C, Nanao MH, Jamin M, Posé D, Thévenon E, Grégoire L, Tichtinsky G, Denay G, Ott F, Peirats-Llobet M *et al.* 2016.** A SAM oligomerization domain shapes the genomic binding landscape of the LEAFY transcription factor. *Nature Communications* **7**: e11222.

**Sessions A, Yanofsky MF, Weigel D. 2000.** Cell–cell signaling and movement by the floral transcription factors LEAFY and APETALA1. *Science* **289**: 779–781.

**Shabek N, Zheng N. 2014.** Plant ubiquitin ligases as signaling hubs. *Nature Structural & Molecular Biology* **21**: 293–296.

**Siriwardana NS, Lamb RS. 2012.** The poetry of reproduction: the role of *LEAFY* in *Arabidopsis thaliana* flower formation. *International Journal of Developmental Biology* **56**: 207–221.

**Souer E, Rebocho AB, Bliek M, Kusters E, de Bruin RAM, Koes R. 2008.** Patterning of inflorescences and flowers by the F-Box protein DOUBLE TOP and the LEAFY homolog ABERRANT LEAF AND FLOWER of petunia. *Plant Cell* **20**: 2033–2048.

**Spoel SH, Mou Z, Tada Y, Spivey NW, Genschik P, Dong X. 2009.** Proteasome-mediated turnover of the transcription coactivator NPR1 plays dual roles in regulating plant immunity. *Cell* **137**: 860–872.

**Thomann A, Brukhin V, Dieterle M, Gheyeselinck J, Vantard M, Grossniklaus U, Genschik P. 2005.** Arabidopsis *CUL3A* and *CUL3B* genes are essential for normal embryogenesis. *Plant Journal* **43**: 437–448.

**Vierstra RD. 2009.** The ubiquitin–26S proteasome system at the nexus of plant biology. *Nature Reviews. Molecular Cell Biology* **10**: 385–397.

**Wagner D, Sablowski RW, Meyerowitz EM. 1999.** Transcriptional activation of APETALA1 by LEAFY. *Science* **285**: 582–584.

**Weber H, Bernhardt A, Dieterle M, Hano P, Mutlu A, Estelle M, Genschik P, Hellmann H. 2005.** Arabidopsis AtCUL3a and AtCUL3b form complexes with members of the BTB/POZ-MATH protein family. *Plant Physiology* **137**: 83–93.

**Weigel D, Alvarez J, Smyth DR, Yanofsky MF, Meyerowitz EM. 1992.** *LEAFY* controls floral meristem identity in Arabidopsis. *Cell* **69**: 843–859.

**Weigel D, Nilsson O. 1995.** A developmental switch sufficient for flower initiation in diverse plants. *Nature* **377**: 495–500.

**Wilkinson MD, Haughn GW. 1995.** UNUSUAL FLORAL ORGANS controls meristem identity and organ primordia fate in Arabidopsis. *Plant Cell* **7**: 1485–1499.

**Winter CM, Austin RS, Blanvillain-Baufumé S, Reback MA, Monniaux M, Wu MF, Sang Y, Yamaguchi A, Yamaguchi N, Parker JE *et al.* 2011.** LEAFY target genes reveal floral regulatory logic, *cis* motifs, and a link to biotic stimulus response. *Developmental Cell* **20**: 430–443.

**Wu M-F, Sang Y, Bezhani S, Yamaguchi N, Han S-K, Li Z, Su Y, Slewinski TL, Wagner D. 2012.** SWI2/SNF2 chromatin remodeling ATPases overcome polycomb repression and control floral organ identity with the LEAFY and SEPALLATA3 transcription factors. *Proceedings of the National Academy of Sciences, USA* **109**: 3576–3581.

**Xu M, Hu T, McKim SM, Murmu J, Haughn GW, Hepworth SR. 2010.** Arabidopsis BLADE-ON-PETIOLE1 and 2 promote floral meristem fate and determinacy in a previously undefined pathway targeting *APETALA1* and *AGAMOUS-LIKE24*. *Plant Journal* **63**: 974–989.

**Yamaguchi N, Wu M-F, Winter CM, Berns MC, Nole-Wilson S, Yamaguchi A, Coupland G, Krizek BA, Wagner D. 2013.** A molecular framework for auxin-mediated initiation of flower primordia. *Developmental Cell* **24**: 271–282.

**Yamashita D, Moriuchi T, Osumi T, Hirose F. 2016.** Transcription factor hDREF is a novel SUMO E3 ligase of Mi2α. *Journal of Biological Chemistry* **291**: 11619–11634.

**Yu Y, Wang SE, Hayward GS. 2005.** The KSHV immediate-early transcription factor RTA encodes ubiquitin E3 ligase activity that targets IRF7 for proteosome-mediated degradation. *Immunity* **22**: 59–70.

**Žádníková P, Simon R. 2014.** How boundaries control plant development. *Current Opinion in Plant Biology* **17**: 116–125.

**Zhang B, Holmlund M, Lorrain S, Norberg M, Bako L, Fankhauser C, Nilsson O. 2017.** BLADE-ON-PETIOLE proteins act in an E3 ubiquitin ligase complex to regulate PHYTOCHROME INTERACTING FACTOR. *eLife* **6**: 1–19.

**Zhang Q, Zhang L, Wang B, Ou CY, Chien CT, Jiang J. 2006.** A hedgehog-induced BTB protein modulates hedgehog signaling by degrading Ci/Gli transcription factor. *Developmental Cell* **10**: 719–729.

**Zhao D, Yu Q, Chen M, Ma H. 2001.** The *ASK1* gene regulates B function gene expression in cooperation with *UFO* and *LEAFY* in *Arabidopsis*. *Development* **128**: 2735–2746.

**Zhao Y, Zhang T, Broholm SK, Tähtiharju S, Mouhu K, Albert V, Teeri TH, Elomaa P. 2016.** Co-opting floral meristem identity genes for patterning of the flower-like Asteraceae inflorescence. *Plant Physiology* **172**: 284–296.

## Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

**Fig. S1** LFY-YFP protein and transcript level in *35S::LFY-YFP* independent $T_1$ plants in wild type and *bop1 bop2* backgrounds of *Arabidopsis thaliana*.

**Fig. S2** Partial suppression of the *35S::LFY* phenotype in a heterozygous *cul3a/+* background of *Arabidopsis thaliana*.

**Fig. S3** Floral abscission defect observed in *Arabidopsis thaliana bop1 bop2* and *cul3a cul3b* mutants.

**Fig. S4** CUL3-BOP2 complex mediates LFY-dependent ubiquitination *in vitro*.

**Table S1** Oligonucleotides used in this study

Please note: Wiley Blackwell are not responsible for the content or functionality of any Supporting Information supplied by the authors. Any queries (other than missing material) should be directed to the *New Phytologist* Central Office.