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An *Arabidopsis* F-box protein acts as a transcriptional co-factor to regulate floral development

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Plants flower in response to both environmental and endogenous signals. The *Arabidopsis* LEAFY (LFY) transcription factor is crucial in integrating these signals, and acts in part by activating the expression of multiple floral homeotic genes. LFY-dependent activation of the homeotic *APETALA3* (*AP3*) gene requires the activity of UNUSUAL FLORAL ORGANS (UFO), an F-box component of an SCF ubiquitin ligase, yet how this regulation is effected has remained unclear. Here, we show that UFO physically interacts with LFY both in vitro and in vivo, and this interaction is necessary to recruit UFO to the *AP3* promoter. Furthermore, a transcriptional repressor domain fused to UFO reduces endogenous LFY activity in plants, supporting the idea that UFO acts as part of a transcriptional complex at the *AP3* promoter. Moreover, chemical or genetic disruption of proteasome activity compromises LFY-dependent *AP3* activation, indicating that protein degradation is required to promote LFY activity. These results define an unexpected role for an F-box protein in functioning as a DNA-associated transcriptional co-factor in regulating floral homeotic gene expression. These results suggest a novel mechanism for promoting flower development via protein degradation and concomitant activation of the LFY transcription factor. This mechanism may be widely conserved, as homologs of UFO and LFY have been identified in a wide array of plant species.

KEY WORDS: *Arabidopsis*, F-box protein, Flower development, Transcriptional co-factor, LEAFY, UFO

INTRODUCTION

Flowering is under the control of multiple endogenous and environmental signals, including hormonal cues, photoperiodic changes and exposure to cold temperatures. In *Arabidopsis*, the *LEAFY* (*LFY*) gene is a key integrator of these signals, in that its expression is upregulated in response to these inputs, and in turn *LFY* acts to coordinately establish the initial expression of the floral homeotic genes (Blazquez and Weigel, 2000; Nilsson et al., 1998; Weigel and Meyerowitz, 1993).

LFY encodes a novel plant-specific transcription factor, and its role in activating floral homeotic genes has been shown to be separable from that of conferring floral meristem identity (Parcy et al., 1998). LFY has been shown to bind to a loosely defined CC(A/T)(A/G)(G/T)G(G/T) consensus site in the regulatory regions of the *APETALA1* (*AP1*), *APETALA3* (*AP3*) and *AGAMOUS* (*AG*) floral homeotic genes (Busch et al., 1999; Lamb et al., 2002; Lohmann et al., 2001; Parcy et al., 1998; Wagner et al., 1999). However, *AP1*, *AP3* and *AG* are expressed in distinct spatial domains, while LFY is expressed throughout the floral meristem, implying that the mode of action of LFY in activating different classes of floral homeotic genes is distinct (Parcy et al., 1998). The spatially and temporally restricted transcriptional activation of both *AP1* and *AG* depends on the concerted action of LFY in combination with other transcription factors. *AG* is expressed in a restricted region consisting of the inner two floral whorls; WUSCHEL (WUS), a homeodomain containing protein that is expressed in the center of the floral meristem, has been shown to directly activate *AG* in conjunction with LFY, resulting in localized expression (Lenhard et al., 2001; Lohmann et al., 2001). *AP1* expression is initially activated throughout the flower in response to LFY activity (Parcy et al., 1998; Wagner et al., 1999), and becomes confined to the first two floral whorls as a consequence of AG-mediated repression in the center of the meristem (Gustafson-Brown et al., 1994).

By contrast, the activation of *AP3* expression in developing petal and stamen primordia requires the activity of the F-box protein *UNUSUAL FLORAL ORGANS* (*UFO*) in conjunction with that of *LFY* (Ingram et al., 1995; Jack et al., 1992; Lee et al., 1997; Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995). Ectopic expression of *UFO* in plants containing a *35S::UFO* transgene results in a dramatic phenotype in which carpels are transformed to stamens, extra petals are formed and leaf margins are serrated (Lee et al., 1997). This gain-of-function phenotype of *UFO* in flowers is due to ectopic *AP3* expression because the phenotype is completely abolished when functional *AP3* is not present (Lee et al., 1997). Every aspect of this gain-of-function phenotype of *UFO* is abolished when *LFY* activity is absent, indicating that the function of *UFO* is dependent on *LFY* activity (Lee et al., 1997). Furthermore, ectopic expression of *LFY* and *UFO* together is sufficient to induce ectopic *AP3* expression, while ectopic expression of *LFY* alone is insufficient (Lee et al., 1997; Parcy et al., 1998). *UFO* is expressed in all shoot meristems in a dynamic pattern, but in the flower the expression domain of *UFO* largely coincides with that of *AP3*, providing regional specification (Lee et al., 1997; Long and Barton, 1998; Samach et al., 1999).

Although *LFY* and *UFO* are both required for the appropriate activation of *AP3* expression, the molecular processes underlying this regulation have been unclear. Most F-box proteins are components of SCF complexes that act as E3 ubiquitin ligases to mark target proteins for proteasome-dependent degradation (Deshaies, 1999). In the SCF E3 ubiquitin ligases, F-box proteins serve as adaptor components, bringing specific substrates to the complex for ubiquitylation and targeting them for degradation (Craig and Tyers, 1999; Feldman et al., 1997; Skowyra et al., 1997).

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UFO has been shown to participate in an SCF^{UFO} complex biochemically and to genetically interact with *Arabidopsis* SCF complex components (Ni et al., 2004; Samach et al., 1999; Wang et al., 2003; Zhao et al., 1999), indicating that UFO has a role in targeted proteolysis.

Some F-box proteins have been shown to regulate the availability of transcription factors or their co-factors for initiating or maintaining transcription. This can occur through direct ubiquitylation and proteasome-mediated degradation of transcription factors (Kodadek et al., 2006; Muratani and Tansey, 2003), or by promoting the degradation of co-factors that can modulate associated transcription factor subcellular localization or activity (Ostendorff et al., 2002; Palombella et al., 1994; Perissi et al., 2004). In particular, controlling the activity and abundance of transcription factors by targeted proteolysis appears to be a mechanism by which cells can effectively switch on and off downstream gene expression. In addition to targeting proteins for destruction, ubiquitylation can also have a non-proteolytic role of modifying protein activity (Kaiser et al., 2000), or physically occlude protein-protein interactions (Horwitz et al., 2007).

The genetic evidence that UFO is required for activating *AP3* expression strongly suggests that the ubiquitin-proteasome system is involved in *AP3* regulation. Here, we demonstrate that UFO acts to modulate *AP3* transcription via regulating LFY transcriptional activity at the promoter. Based on our observations, we propose that UFO, as a component of an E3 ubiquitin ligase, functions as a co-factor for LFY-induced *AP3* floral homeotic gene transcription and regulates the activity of the LFY transcription factor in a proteasome-dependent manner.

MATERIALS AND METHODS

Plant material and transformation

Plants were grown in 3:1 vermiculite: soil at 22°C under 16-hour light/8-hour dark conditions. For dose studies, *lfy-26* homozygotes were crossed to homozygous *35S::UFO-myc* plants. Homozygotes for *35S::UFO-myc* were selected in the F2 generation. Siblings from the F4 generation segregating for *lfy-26* in the *35S::UFO-myc* homozygous background were assessed for their phenotypes. For *35S::UFO-Myc; 35S::LFY-FLAG; ufo-2*, each plant was subjected to western blot analysis to assay expression levels of epitope tagged proteins. Plants expressing both proteins at high levels were used for co-immunoprecipitations. For *35S::LFY-GR; 35S::UFO; hlr-1*, kanamycin was used to identify the appropriate genotype that show a halted root mutant phenotype. Transgenic lines were generated using the floral dip method (Clough and Bent, 1998). *35S::UFO-myc* plants were generated previously (Wang et al., 2003); *lfy* and *ufo* mutants were obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus, OH) and *hlr-1* seeds were a kind gift from Dr K. Okada, Kyoto University.

DNA constructs

pUFOA and pLFYX used in EMSA assays and GST pull-down assays have been reported (Lamb et al., 2002). Yeast constructs AD-LFY and BD-UFO were generated by inserting the LFY open reading frame into pGAD424 and that of UFO into pGBT9. BD-ΔFUFO was constructed by excising the F-box of UFO (amino acids 1-87) using *Nco*I and *Xho*I sites from BD-UFO and religated. Truncated versions of LFY were constructed using Expand High Fidelity PCR (Roche Applied Science, Indianapolis, IN). *35S::LFY-FLAG* was constructed by inserting three FLAG tags (DYKDDDDK) at the C terminus of LFY. To generate *SRDX* fused to *UFO* in frame, the ORF of *UFO* was cloned into the Gateway entry vector pDONR201 and then recombined into the destination vector pH35GEAR [a gift from Taku Demura (Kubo et al., 2005)], resulting in *35S::UFO-SRDX*. The negative control (*35S::UFO-mSRDX*) was generated by replacing the SRDX domain with a FLAG tag in *35S::UFO-SRDX*. This results in a UFO fusion protein containing an additional 19 amino acids (PAFLYKVVDNSDYKDDDKA; the FLAG tag is underlined; remaining sequences derived from vector) at the C-terminal end. *35S::UFO-SRDX* contains an additional 24 amino acids (AFLYKVVVISRPLDLDLELRLGFA; SRDX domain is underlined; remaining sequences derived from vector). All binary expression constructs were transferred to *Agrobacterium tumefaciens* strain GV3101 by electroporation.

Proteins for EMSA were transcribed and translated in vitro using wheat germ extract (Promega, Madison, WI). Preparation of DNA probes, binding reactions and gel running conditions were as described previously (Hill et al., 1998; Lamb et al., 2002).

Yeast two-hybrid assays

In frame fusion constructs were generated in pGAD424 or pGBT9 (Clontech, Palo Alto, CA) and transformed into the yeast strain Y190. *lacZ* liquid assays were performed as described previously (Tan and Irish, 2006).

In vitro binding assays

GST-LFY and GST constructs were expressed in *Escherichia coli* strain BL21 (DE3) codon plus (Stratagene, La Jolla, CA) and affinity purified using glutathione sepharose 4B beads (Amersham Biosciences, Piscataway, NJ). Approximately 1 μg of GST-LFY and GST-LFY-N2, based on spectrophotometric measurements, were incubated overnight with [^{35}S] Met-labeled in vitro transcribed and translated UFO at 4°C in 0.2 ml of buffer A [100 mM NaCl, 50 mM Tris-Cl (pH 7.5), 0.1 mM EDTA, 0.1 mM EGTA, 50 mM NaF, 0.2% Triton X-100, 0.1% β-mercaptoethanol, complete protease inhibitor (Roche, Applied Science, Indianapolis, IN) and 1 mM phenylmethylsulfonyl fluoride]. The beads were washed four times with ice-cold buffer B [100 mM NaCl, 50 mM HEPES (pH 7.5), 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and complete protease inhibitor] at 4°C. The washed beads were boiled in SDS sample buffer, and proteins were separated by SDS-PAGE. Gels were fixed, dried and exposed to X-ray film.

Immunoprecipitation

Total protein from inflorescence tissue was extracted using an extraction buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM $MgCl_2$, 10% glycerol, 0.1% NP-40, 1 mM phenylmethylsulfonyl fluoride, and complete protease inhibitor (Roche, Applied Science, Indianapolis, IN). Immunoprecipitation was performed using anti-FLAG M2 agarose (Sigma, St Louis, MO) at 4°C overnight. After washing four times with extraction buffer lacking NP-40, proteins were eluted using 3XFLAG (Sigma, St Louis, MO). Immune complexes were analyzed by SDS-PAGE and immunoblotted using anti-FLAG (M2, Sigma, St Louis, MO) or anti-Myc (9E10, Covance, Philadelphia, PA) antibodies and were detected using an ECL kit (Amersham Biosciences, Buckinghamshire, UK). To detect LFY ubiquitylation, immunoprecipitations were performed as above, except that the extraction buffer contained 20 μM MG132, and incubated for 3 hours at 4°C. The pellet was washed with extraction buffer four times and eluted using 3XFLAG. Samples were fractionated by SDS-PAGE and immunoblotted with either anti-FLAG (M2, Sigma, St Louis, MO) or anti-ubiquitin (Sigma, St Louis, MO) antibodies. For anti-ubiquitin immunoblot, the membrane was pre-treated with denaturing buffer [6 M guanidine HCl, 20 mM Tris-HCl (pH 7.5), 5 mM β-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride] as described (Miller et al., 2004).

Chromatin immunoprecipitation

The procedure essentially followed (Wang et al., 2002) and (Ito et al., 1997). Approximately 5 g of inflorescence tissue from either *UM* or *UM; lfy-26* was used as starting material. Immunoprecipitation (IP) was performed using 30 μl of anti-Myc affinity matrix (Covance, Philadelphia, PA). For control IP, the same amount of normal mouse IgG serum conjugated to agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) was used. Inflorescence tissue from either *35S::LFY-FLAG* or *35S::LFY-FLAG;ufo-2* was used to IP the LFY-chromatin complex using 30 μl of anti-FLAG resin (Sigma, St Louis, MO). LFY-FLAG was eluted from the resin using 3XFLAG peptides. Amplification of genomic fragments was performed following standard semi-quantitative PCR conditions, with 28 to 35 cycles of amplification.

Chemical treatments

Seven day-old seedlings of different genotypes were transferred to solid MS media containing 1 μM dexamethasone (DEX, Sigma, St Louis, MO) or 0.01% ethanol for mock treatment. Epoxomicin (10 μM) (Sigma, St Louis, MO) in liquid MS media was added to DEX containing MS and vacuum infiltrated for 1 minute. DMSO was used as mock control. After 4 hours of treatment, seedlings were harvested in liquid nitrogen and used for RNA preparation. For epoxomicin treatment, inflorescences were treated with 1 μM epoxomycin in 1/2 MS, 0.02% silwet for 4 hours.

Quantitative RT-PCR

RNA was extracted from seedlings using Trizol (GibcoBRL, Frederick, MD). cDNA was synthesized using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). Quantitative real-time PCR was performed in 25 μl using the QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA) on an ABI Prism 7900 HT Sequence Detector (Applied Biosystems, Foster City, CA). Reaction conditions were: 95°C for 15 minutes, followed by 40 cycles of 94°C for 15 seconds, 53°C for 30 seconds and 72°C for 30 seconds. Primer sequences for *AP3* have been reported (Lamb et al., 2002). Triplicate assays were carried out using at least two independent RNA samples. The amount of mRNA was quantified using DART-PCR 1.0 (Peirson et al., 2003) or $2^{-\Delta\Delta Ct}$ and normalized either to the value of eukaryotic translation elongation factor-1α (EF-1α) or to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). PCR products were subjected to agarose gel electrophoresis to test the specificity of the amplification.

RESULTS

The stoichiometry of UFO and LFY suggests a direct physical interaction

Plants containing an epitope tagged version of *UFO* driven by the 35S promoter, *35S::UFO-Myc* (Wang et al., 2003), displayed an ectopic petal and stamen phenotype (Fig. 1A,B) and serrated leaves (Fig. 1F, left). This phenotype is identical to that produced by *35S::UFO* (Lee et al., 1997), indicating that the epitope tag does not interfere with UFO activity. In addition, the complete elimination of LFY function in the *35S::UFO-Myc* plants also resulted in a *lfy* mutant phenotype, with a complete transformation of flowers to shoots (Fig. 1A, arrows) or intermediate structures between flower and shoot (Fig. 1D). We observed that when *LFY* is heterozygous in the *35S::UFO-Myc* background, the plants showed suppression of the ectopic *35S::UFO-Myc* phenotype (Fig. 1A,C,E,F). Unlike *35S::UFO-Myc* plants, which are completely infertile because of a complete transformation of carpels to stamenoid organs (Fig. 1A,B,E), *35S::UFO-Myc; lfy-26*/+ flowers produced short curved siliques containing viable seeds (Fig. 1A,E). Most *35S::UFO-Myc; lfy-26*/+ flowers appeared similar to wild type with four petals and six stamens (Fig. 1C). Serration of leaf margins, observed in *35S::UFO-Myc* plants, was also reduced in the *35S::UFO-Myc; lfy-26*/+ plants (Fig. 1F, middle), consistent with a proposed role for LFY during vegetative development (Lee et al., 1997). These genetic data indicate that there are dose dependent effects of *LFY* on the *35S::UFO-Myc* phenotype, suggesting that LFY and UFO physically interact in a complex.

LFY physically interacts with UFO

To demonstrate explicitly a physical interaction between LFY and UFO, we carried out several assays. Electrophoretic mobility shift assays (EMSA) have shown that LFY binds directly to sequences in the *AP3* promoter (Lamb et al., 2002) (Fig. 2A). We showed that EMSA using both LFY and UFO proteins resulted in a supershift of the DNA/protein complex, even though UFO itself did not show any DNA-binding affinity for *AP3* promoter sequences (Fig. 2A). These results indicate that UFO and LFY can directly and physically interact, at least within the context of *AP3* promoter sequences. To

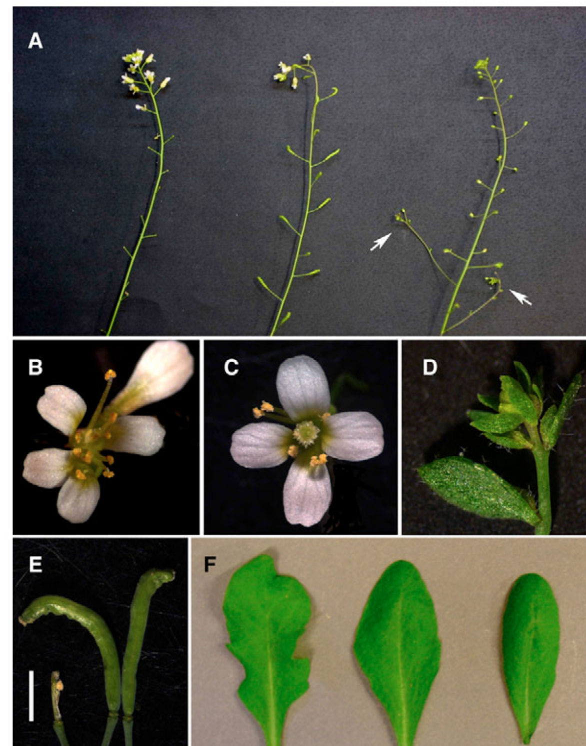

Fig. 1. The stoichiometry of LFY and UFO is important in flower development. (**A**) Inflorescences of *35S::UFO-Myc*, *35S::UFO-Myc; lfy-26*/+ and *35S::UFO-Myc; lfy-26* plants (from the left). Most flowers of *35S::UFO-Myc; lfy-26*/+ produce siliques, whereas *35S::UFO-Myc* flowers are completely infertile. Arrows indicate extra co-florescences on *35S::UFO-Myc; lfy-26* plants. Representative plants were siblings obtained from a segregating F4 population. (**B**) *35S::UFO-Myc* flower with extra petals and stamens. Carpels are transformed to stamenoid organs. (**C**) A representative *35S::UFO-Myc;lfy-26*/+ flower; most such flowers are phenotypically normal, except that they produce curved siliques. (**D**) A representative *35S::UFO-Myc; lfy-26* flower that is indistinguishable from *lfy-26* mutant flowers. (**E**) Comparison of stamenoid fourth whorl organs from *35S::UFO-Myc* (left) and two siliques from *35S::UFO-Myc; lfy-26*/+plants (right). Scale bar: 2 mm. (**F**) Comparison of a rosette leaf from *35S::UFO-Myc* (left), *35S::UFO-Myc; lfy-26*/+ (middle) and *35S::UFO-Myc; lfy-26* plants (right).

examine whether such interactions were dependent on the presence of DNA, we also performed GST-pull down assays. As shown in Fig. 2B, a GST-LFY fusion protein could successfully pull down radiolabeled UFO, whereas GST alone could not. In addition, yeast two-hybrid assays that we carried out also demonstrated that LFY and UFO can physically associate in the absence of *AP3* promoter DNA. LFY directly interacted with UFO when UFO was fused to the GAL4 DNA-binding domain and LFY was fused to the GAL4 activation domain (Fig. 2C). However, the reciprocal fusion of the GAL4 domain to LFY and UFO did not result in a positive interaction (Fig. 2C). As the conformation of an unoccupied F-box has been suggested to occlude protein-protein interaction domains (Deshaies, 1999), we reasoned that the protein-protein interaction domain of UFO would be more accessible when the F-box domain of the protein was deleted. As expected, a ΔFUFO construct that lacks the F-box showed a much more robust interaction with LFY in yeast, irrespective of whether ΔFUFO was fused to the Gal4

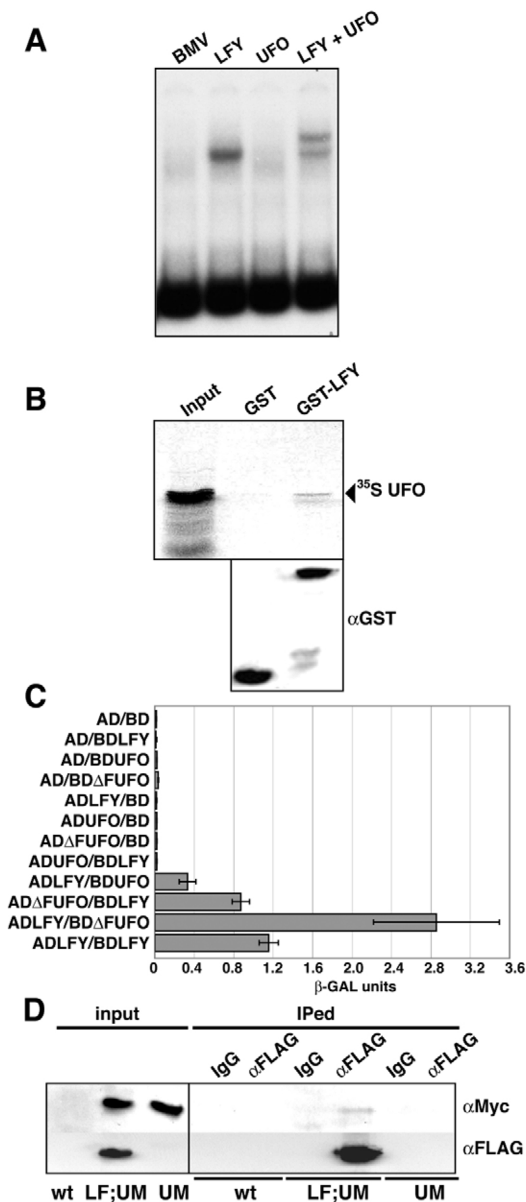

Fig. 2. LFY and UFO physically interact. (**A**) UFO induces a supershift of a LFY-DNA complex. Electrophoretic mobility shift assay (EMSA) using an *AP3* promoter sequence. BMV, non-specific brome mosaic virus control; LFY, in vitro transcribed and translated LFY protein; UFO, in vitro transcribed and translated UFO protein; LFY+UFO lane shows a supershift of the LFY-DNA complex, while UFO alone does not bind to the *AP3* promoter sequence, indicating that UFO binds to the LFY-DNA complex. (**B**) Upper panel: GST pull-down assay showing interaction between a bacterially produced GST-LFY fusion protein and ^{35}S-labeled UFO protein; GST alone does not show an interaction with UFO. Input lane represents 10% of total protein. Lower panel shows same blot probed with αGST antibody, demonstrating equivalent loading of GST and GST-LFY lanes. (**C**) Interaction of LFY and UFO in yeast two-hybrid assays. The AD-LFY/BD-UFO interaction shows a significant increase in β-gal activity over background controls. This interaction is enhanced 8.6-fold when the F-box is deleted in the AD-LFY/BD-ΔFUFO combination compared with that of AD-LFY/BD-UFO. AD-ΔFUFO and BD-LFY also shows a significant interaction, while AD-UFO and BD-LFY shows a background level of β-gal activity. AD, Gal4 activation domain; BD, Gal4 DNA-binding domain. Bars represent mean±s.e.m. for five replicates. (**D**) Co-immunoprecipitation of UFO-Myc with LFY-FLAG in planta. Protein from wild-type*, 35S::LFY-FLAG, 35S::UFO-Myc; ufo-2 (LF; UM)* or *35S::UFO-Myc (UM)* floral buds were used to precipitate the LFY immune complex using α-FLAG. For control immunoprecipitations, normal mouse IgG serum conjugated to agarose beads was used. Myc-tagged UFO specifically co-precipitated with LFY-FLAG from *35S::LFY-FLAG, 35S::UFO-Myc* tissue only.

activation domain or to the Gal4 DNA-binding domain (Fig. 2C). We also found that LFY protein can homodimerize in yeast cells (Fig. 2C), while UFO does not (data not shown).

To test whether or not this interaction can be observed in a physiologically relevant context, we examined the ability of LFY and UFO to interact in inflorescence tissue. We generated transgenic plants harboring epitope tagged versions of both LFY and UFO. Because *35S::UFO-Myc; 35S::LFY-FLAG* plants arrest at the seedling stage in a manner similar to that of *35S::UFO; 35S::LFY* plants (Parcy et al., 1998) (and data not shown), we used *35S::LFY-FLAG; 35::UFO-Myc; ufo-2* (*LF; UM*) plants as a source of inflorescence tissues. These *LF; UM* plants produce flowers with petals or petaloid organs, indicating that both transgenes are functional, and that the dose of *UFO* is appropriate to bypass lethality (see Fig. S1 in the supplementary material). For co-immunoprecipitations, we used a line in which both the *35S::LFY-FLAG* and *35S::UFO-Myc* transgenes showed robust expression (Fig. 2D, input lanes). We performed immunoprecipitations with an anti-FLAG antibody to pull down the LFY immune complex and tested whether or not UFO was co-immunoprecipitated. As shown in Fig. 2D, UFO-Myc was detected in the anti-FLAG immunoprecipitated lane from *LF; UM* tissue, indicating that UFO co-immunoprecipitates with LFY. This interaction was weak but detectable, suggesting that UFO-LFY interactions may be transient. Neither control IgG immunoprecipitations nor immunoprecipitations from plants expressing only epitope-tagged UFO (*UM*) showed any UFO bands, indicating that this interaction is specific. Together, these observations demonstrate that UFO physically interacts with LFY both in vitro and in vivo.

The C-terminus of LFY is required for interaction with UFO

Phylogenetic analyses of LFY orthologs identified conserved N-terminal and DNA-binding C-terminal domains (Maizel et al., 2005). Nonsense mutations in *LFY*, even those located within the C-terminus [e.g. *lfy-26* that lacks the last 48 amino acids (Lee et al., 1997; Maizel et al., 2005)], cause strong *lfy* mutant phenotypes, implying that the C-terminal domain is crucial for LFY function. To determine which domains in LFY are required for the interaction with UFO, we generated four truncated versions of LFY: LFYN1 (amino acids 1-141), LFYC1 (amino acids 142-420), LFYN2 (amino acids 1-375, equivalent to the truncated mutant form found in *lfy-26*) and LFYC2 (amino acids 376-420) (Fig. 3A). Yeast two-hybrid assays revealed that LFYN1 failed to interact with ΔFUFO, while LFYC1 still retained the interaction albeit at somewhat reduced levels (Fig. 3B), indicating that the C-terminal domain of LFY is crucial for interaction with UFO and that the N-terminal domain may stabilize the interaction. Interestingly, LFYN2 did not show any interaction with ΔFUFO. We confirmed the abrogation of

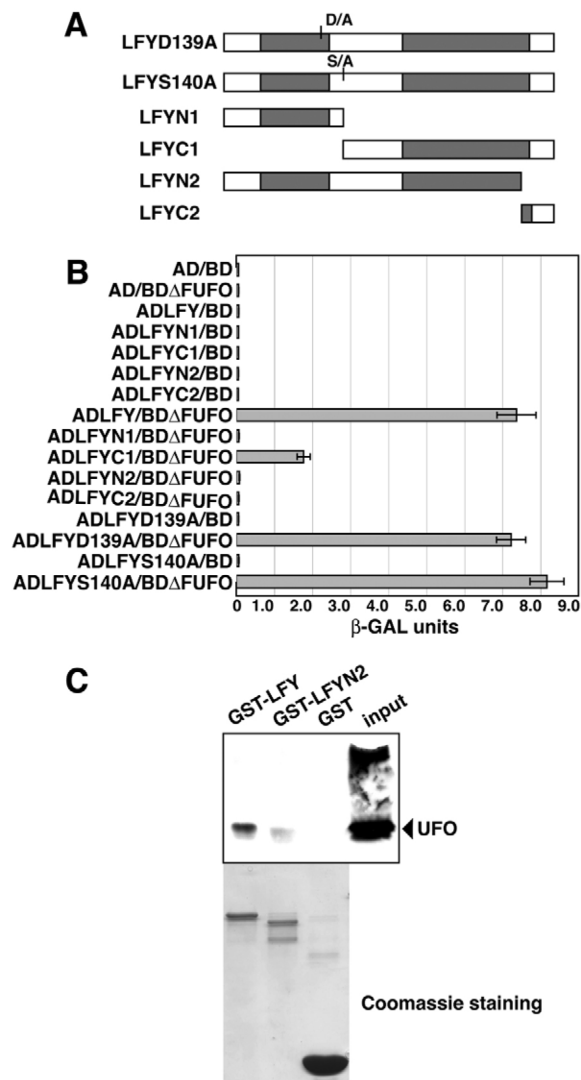

Fig. 3. Mapping of the LFY interaction domain. (**A**) Diagram of the mutated and truncated forms of LFY. Gray boxes indicate the conserved domains at the N terminus and C terminus. Either D139 or S140 were mutated to alanine and used for yeast two-hybrid assays. Four different truncated versions of LFY, LFYN1 (amino acids 1-141), LFYC1 (amino acids 142-420), LFYN2 (amino acids 1-375) and LFYC2 (amino acids 376-420) are shown. (**B**) Yeast two-hybrid assays testing the interaction between various LFY truncations/mutants and ΔFUFO. Quantitative measurements of β-GAL activities (average of five independent assays) are indicated. LFYN2 fails to interact with ΔFUFO, while LFYC1 retains the interaction. Neither of the mutant forms D139 nor S140 affected the interaction with ΔFUFO. Bars represent mean±s.e.m. for the five replicates. (**C**) GST pull-down assays performed with bacterially expressed GST fusion proteins and inflorescence protein extracts from *35S::UFO-Myc* plants. Top panel, western blot probed with anti-Myc antibody. Bottom panel, Coomassie Blue stained gel showing GST fusion proteins used in the assays. LFYN2 does not pull down UFO as efficiently as does full-length LFY from plant extracts.

the interaction of LFYN2 with full-length UFO using GST pull-down assays, which were performed with bacterially produced GST fusion proteins and protein extracts from inflorescence tissues of *35S::UFO-Myc* plants (Fig. 3C). Full-length LFY protein efficiently pulled down UFO protein from plant extracts, while LFYN2 failed to do so. Considering that a LFYN2 construct has also been shown to lack DNA-binding activity (Maizel et al., 2005), it appears that an intact C terminus is important for LFY function in two ways: conferring stable DNA binding affinity and interacting with UFO.

Interestingly, LFY contains a sequence motif (DSGTH; amino acids 139-143) similar to the DSGXS ‘destruction box’ motif found in substrates of the F-box protein β-TRCP (Winston et al., 1999; Yaron et al., 1997). We tested whether the motif present in LFY can serve as the recognition site for UFO by mutagenizing the aspartic acid or serine residue to alanine (Fig. 3A). Yeast two-hybrid assays showed that mutation of either D139 or S140 had no effect on LFY interaction with either UFO or ΔFUFO (Fig. 3B). This observation indicates that the cryptic destruction box present in LFY is not required for recognition by the F-box protein UFO.

UFO is recruited to the *AP3* promoter via LFY

The observation of physical interaction between LFY and UFO led us to test whether UFO could potentially act as a transcriptional co-factor and be recruited to the *AP3* promoter through its physical association with LFY. Two *AP3* promoter elements (termed DEE and PEE) have been shown to be essential for establishing the initial domain of *AP3* expression (Hill et al., 1998). LFY has been previously shown to bind to the DEE region of the *AP3* promoter in vitro (Lamb et al., 2002). We first sought to confirm the LFY-*AP3* promoter association in vivo using chromatin immunoprecipitation (ChIP). ChIP carried out using inflorescence tissue from *35S::LFY-FLAG* plants showed that both the DEE and PEE sequence elements are enriched in the immunoprecipitated chromatin when compared with controls (Fig. 4A,B). We confirmed that we could recapitulate the interaction of LFY protein with other known DNA targets such as the *AP1* promoter (William et al., 2004), as *AP1* promoter sequences were enriched in our ChIP assays (Fig. 4B). ChIP assays from inflorescence tissues of *35S::LFY-FLAG; ufo-2* plants also resulted in enrichment of the promoter region tested (Fig. 4B), indicating that functional UFO is not required for LFY binding to target sequences, although it may facilitate such binding. Control IPs or ChIP assays performed using wild-type tissue showed no significant enrichment of the promoter regions tested, nor did the control Mu genomic region show specific enrichment, indicating that the binding of LFY to the promoter fragments of *AP1* and *AP3* was specific (Fig. 4B).

To examine if UFO can be recruited to the *AP3* promoter, we performed ChIP assays using inflorescence tissues of *35S::UFO-Myc* plants and examined whether *AP3* promoter fragments were enriched in the immunoprecipitated chromatin. ChIP analyses (Fig. 4C) showed that UFO associates with the *AP3* promoter and localizes to both *AP3* promoter elements. Furthermore, we observed that the association of UFO with the *AP3* promoter was abolished when ChIP was performed using extracts from *lfy-26* mutant plants harboring the *35S::UFO-Myc* transgene (Fig. 4C). As this association is dependent on the presence of functional LFY protein, these results indicate that UFO is recruited to the *AP3* promoter through its interaction with LFY.

UFO functions as a transcriptional cofactor

To assay the ability of *UFO* to function as a transcriptional co-factor of LFY in planta, we fused the strong SRDX transcriptional repressor domain that contains an EAR motif (Hiratsu et al., 2003) to *UFO* and introduced it into *Arabidopsis* plants under the control of the constitutive *35S* promoter. If *UFO* acts as a LFY transcriptional co-factor, then *35S::UFO-SRDX* should confer dominant repressor activity to the transcription events that *LFY*

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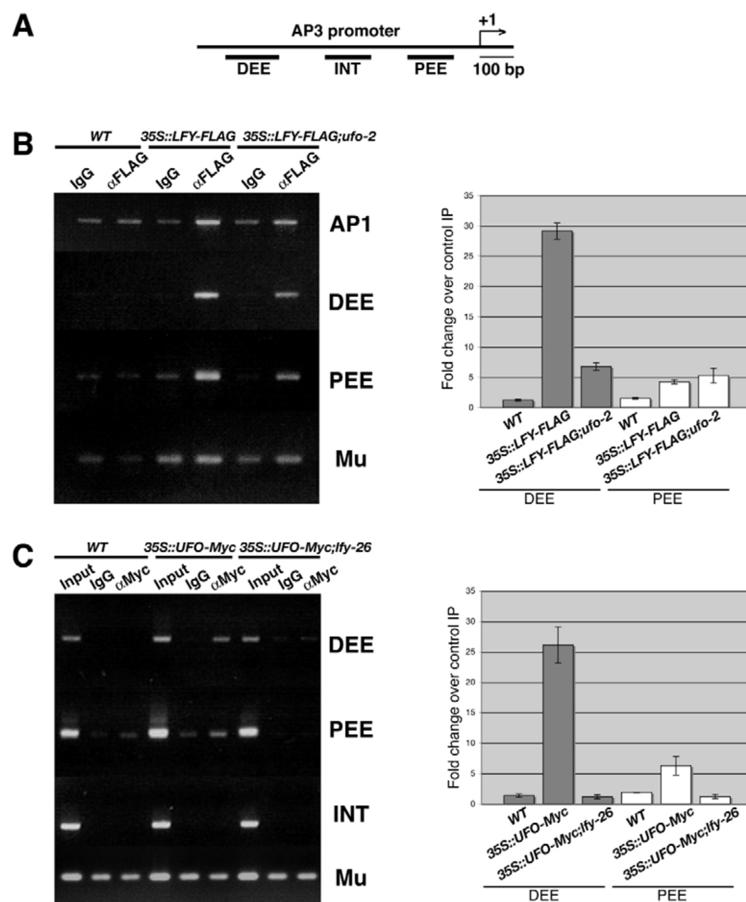

Fig. 4. Recruitment of LFY and UFO to the *AP3* promoter. (**A**) The *AP3* genomic region. Three different regions of the *AP3* promoter, DEE (distal early element), PEE (proximal early element) and INT (inter-region between DEE and PEE) (Hill et al., 1998) are illustrated. (**B**) (Left) Chromatin immunoprecipitation (ChIP) was performed with anti-FLAG antibody or mouse normal IgG serum from inflorescence tissue of plants of the indicated genotypes. Promoter regions from *AP1* and *AP3* were amplified using PCR as indicated. A region of the *Mu* transposon was used as a positive control for amplification. LFY specifically associates with both the DEE and PEE elements of the *AP3* promoter, as well as with the *AP1* promoter fragment. The *ufo-2* mutation does not compromise the ability of LFY to bind to target sequences. (Right) DEE and PEE levels were normalized to Mu and the fold change of experimental IP over IgG control IP is indicated. The values are mean±s.e.m. from three PCR experiments. (**C**) (Left) ChIP was carried out using inflorescence tissues obtained from genotypes as indicated. Chromatin immunoprecipitations carried out using anti-Myc antibody or normal mouse IgG serum. UFO specifically associates with both *AP3* promoter regions, DEE and PEE; however, the presence of the *lfy-26* mutation abolished these interactions, indicating that LFY is required for UFO to associate with *AP3* promoter sequences. (Right) Quantitation as in B.

governs, resulting in a phenotype similar to that of *lfy* mutants. However, if *UFO* does not act via modulating transcription, the *35S::UFO-SRDX* construct should generate a phenotype more similar to that of a *UFO* gain-of-function transgene. We also generated *35S::UFO-mSRDX* transgenic plants in which the SRDX domain has been replaced with an epitope tag of similar size; these plants were used to control for the possibility that a chimeric UFO protein would non-specifically abrogate LFY activity.

As expected, we observed that ten out of 30 independent primary *35S::UFO-SRDX* transformants showed a range of *lfy*-like mutant phenotypes (Fig. 5). In addition, one line appeared indistinguishable from the strong *ufo*-2 mutant (Fig. 5F), whereas none of the transgenic lines showed the gain-of-function phenotype of *UFO* (Fig. 5; see Fig. S2 in the supplementary material). As is prominently seen in *lfy* mutants, these ten lines showed defects in petal and stamen development (Fig. 5; see Fig. S2). Furthermore, we also observed a complete or partial transformation of flowers to inflorescence shoots, which is a hallmark of *lfy* mutants (Huala and Sussex, 1992; Schultz and Haughn, 1991; Weigel et al., 1992). For example, two severe lines had up to six secondary inflorescences (data not shown) and most lines harbored intermediate structures between inflorescence shoots and flowers (Fig. 5B-D). These intermediate structures were often subtended by bracts (Fig. 5I, arrow), with leaf-like organs arranged in a spiral pattern rather than in a whorl (Fig. 5B-D) and internode elongation was also often observed (Fig. 5C,D). The only difference we observed in *35S::UFO-SRDX* plants compared with *lfy* mutants is that secondary flowers rarely form in the axils of leaf-like organs in *35S::UFO-SRDX* plants when compared with *lfy* mutants, which is consistent with the proposed role of LFY in functioning as a transcriptional repressor in inhibiting secondary flower formation (Parcy et al., 2002). Later in development, the *35S::UFO-SRDX* flowers tended to terminate with fused carpelloid leafy organs (Fig. 5E), which is also a typical characteristic of *lfy* mutants (Huala and Sussex, 1992; Schultz and Haughn, 1991; Weigel et al., 1992). In most lines, the transformation of flowers to inflorescence shoots was less severe in later arising flowers (Fig. 5D; see Fig. S2A,B). RT-PCR analyses showed that none of these lines had a reduction in endogenous *UFO* transcript levels when compared with wild type, indicating that the *35S::UFO-SRDX* phenotypes are not caused by co-suppression of endogenous *UFO* expression (data not shown). Furthermore, none of plants expressing *35S::UFO-mSRDX* showed any *lfy*-like mutant phenotypes, indicating that the mSRDX fusion does not confer a dominant-negative effect. Rather, these control *35S::UFO-mSRDX* plants showed a typical *UFO* overexpression phenotype. Together, these results demonstrate that the transformations seen in the *35S::UFO-SRDX* plants reflected the dominant transcriptional repressor activity conferred by the fusion of the SRDX domain to UFO. The observed dominant-negative effect of the UFO-SRDX protein on LFY activity supports the idea of a direct physical interaction between LFY and UFO, and suggests that UFO acts as a LFY transcriptional co-factor.

The two most severe *35S::UFO-SRDX* lines showed more drastic transformations than the strongest *lfy* alleles characterized to date in terms of floral organ identity defects. These two severe lines produced lateral structures consisting of only reiterating leaf-like organs with stipules arranged in a spiral pattern (Fig. 5G, see Fig. S2C in the supplementary material). This severe phenotype was

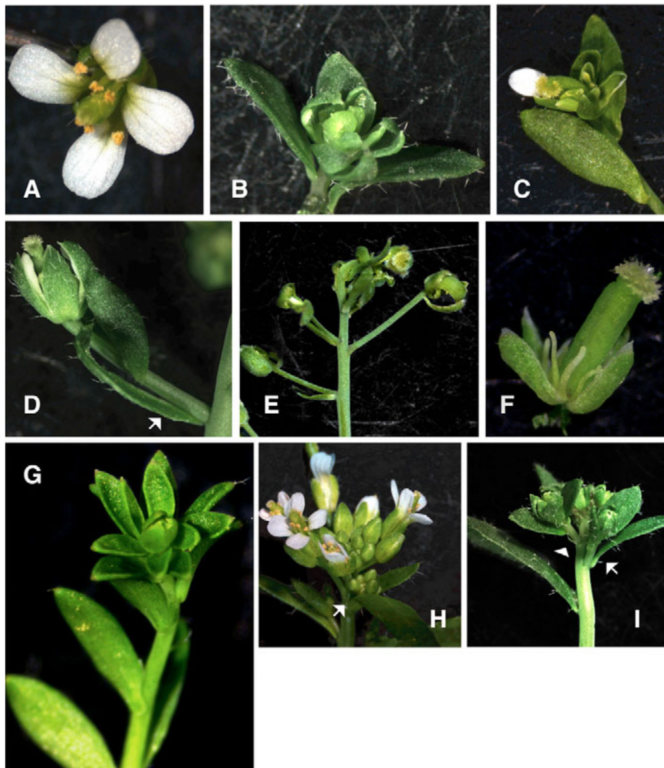

Fig. 5. UFO-SRDX represses endogenous LFY activity. (A,H) WT (L. *er*) plants. (B-G,I) *35S::UFO-SRDX* plants. (**A**) A wild-type flower. (**B**) The first arising flower-like structure in a transgenic *35S::UFO-SRDX* line. The structure is subtended by a bract and consists of leafy organs, sepal-like organs and carpelloid sepals arranged in a spiral. (**C**) The fourth structure produced on the primary inflorescence. Organs are arranged in a spiral pattern. Occasionally, such flowers produce petals with reduced number and size. The flowers are infertile. (**D**) A later-arising flower with floral organs arranged in a whorl and subtended by bracts (arrow). The bracts are spirally arranged and show internode elongation. (**E**) A transgenic primary inflorescence in which the apex terminates with fused carpelloid sepal-like organs. Even apical flowers are subtended by bracts. (**F**) A representative *35S::UFO-SRDX* flower from the line that resembles *ufo-2*. Like *ufo-2* flowers, petals and stamens are absent or reduced in number and size or are filamentous. Carpelloid sepals and mosaic organs are often also observed. (**G**) A lateral structure from one of the most severe transgenic lines. No floral organs are produced; rather, only leaf-like organs arise in a spiral phyllotaxy with internodes elongated resembling *ap1 lfy* double mutants. (**H**) A wild-type inflorescence subtended by cauline leaves (arrow). (**I**) An inflorescence from a *35S::UFO-SRDX* plant. Flowers are subtended by prominent bracts (arrow) or stipules (arrowhead). Only inflorescences but not flowers are subtended by cauline leaves in wild type (H).

maintained throughout the inflorescence, and was not ameliorated over time. This indicates that the specification of floral organ identity was almost completely repressed in those lines. This phenotype resembled that found in *ap1 lfy* double mutants (Bowman et al., 1993; Huala and Sussex, 1992; Weigel et al., 1992), and suggests the possibility that *35S::UFO-SRDX* not only reduces the transcriptional activity of LFY, but also potentially of AP1.

To examine whether these severe phenotypic defects are correlated with transgene expression levels, we quantified transcript levels of the UFO-SRDX transgene using quantitative RT-PCR. Indeed, the two most severe lines harboring lateral structures with only leafy organs (Fig. 5G) showed the highest levels of UFO-SRDX transcripts (see Fig. S2D in the supplementary material). In addition, the two strong lines that showed *lfy* mutant phenotypes (Fig. 5B-E; see Fig. S2D in the supplementary material) produced a few progeny that segregated in a 1:2 ratio of plants appearing identical to the most severe lines of the primary transformants when compared with parental lines, which was consistent with homozygous and hemizygous segregants for the transgene. Therefore, expression studies of the transgene and progeny testing strongly suggest that all the observed phenotypic defects described above were due to a strong dominant repressive effect of the UFO-SRDX transgene.

Proteasome activity is required for ectopic *AP3* induction

Given that the F-box protein UFO associates with the *AP3* promoter and functions as a transcriptional co-factor of LFY, it is probable that UFO mediates transcriptional regulation in a proteasome-dependent manner. To test if 26S proteasome activity is required for *AP3* expression in vivo, we used an inducible form of LFY, *35S::LFY-GR* (Wagner et al., 1999) and a proteasome mutant, *halted root* (*hlr*) (Ueda et al., 2004). This inducible form of LFY can activate *AP3* transcription in seedlings upon DEX treatment only in the presence of UFO (Lamb et al., 2002). The proteasome subunit Rpt2a mutant *hlr* has been shown to have reduced proteasome activity during seedling development, and thus fails to degrade the substrate of the well characterized SCF complex SCF^{TIR1} (Ueda et al., 2004). Therefore, we introduced *35S::LFY-GR* and *35S::UFO* into a *hlr* mutant background and then tested for the requirement of proteasome activity for LFY and UFO to activate *AP3* transcription in seedlings. *35S::LFY-GR; 35S::UFO; hlr-1* seedlings were grown without DEX for 7 days, treated with 1 μM DEX for 16 hours, and then harvested and the relative levels of *AP3* expression assayed using qRT-PCR. We used *35S::LFY-GR; 35S::UFO* seedlings as a positive control; DEX treatment resulted in the induction of *AP3* expression as previously reported (Lamb et al., 2002) (Fig. 6A). However, *AP3* expression was not induced in the *hlr* mutant background, even in the presence of both LFY and UFO, indicating that proteasome activity is required for *AP3* transcription to ensue (Fig. 6A).

To independently confirm the requirement of proteasome activity for the induction of *AP3* expression in seedlings, we also used epoxomicin, a potent inhibitor of the proteolytic activity of the 20S core component of the proteasome (Meng et al., 1999). We observed that epoxomicin treatment reduced the level of *AP3* transcripts induced by DEX (Fig. 6B) up to 64% and 40 % in two independent biological replicates, respectively. These results indicate that proteasome activity is required for *AP3* to be fully induced in seedlings by LFY and UFO. These observations support the idea that UFO acts to target proteins for 26S proteasome mediated degradation, and that such degradation is a requirement for *AP3* transcription.

To test whether UFO can act to post-translationally modify LFY, we examined the ubiquitylation status of LFY protein. When LFY-FLAG protein was subjected to SDS-PAGE, we detected the fusion protein at the expected size, but also saw a faint smear of cross-reacting higher molecular weight proteins (Fig. 6C, lane 1), suggesting that LFY may be ubiquitylated in vivo. The abundance of these high molecular weight proteins is somewhat reduced in a *ufo-2* mutant background, suggesting that UFO may act to modify post-translationally a subset of LFY proteins in vivo (Fig. 6C, lane 2; Fig. 6D). We further showed that the levels of high molecular

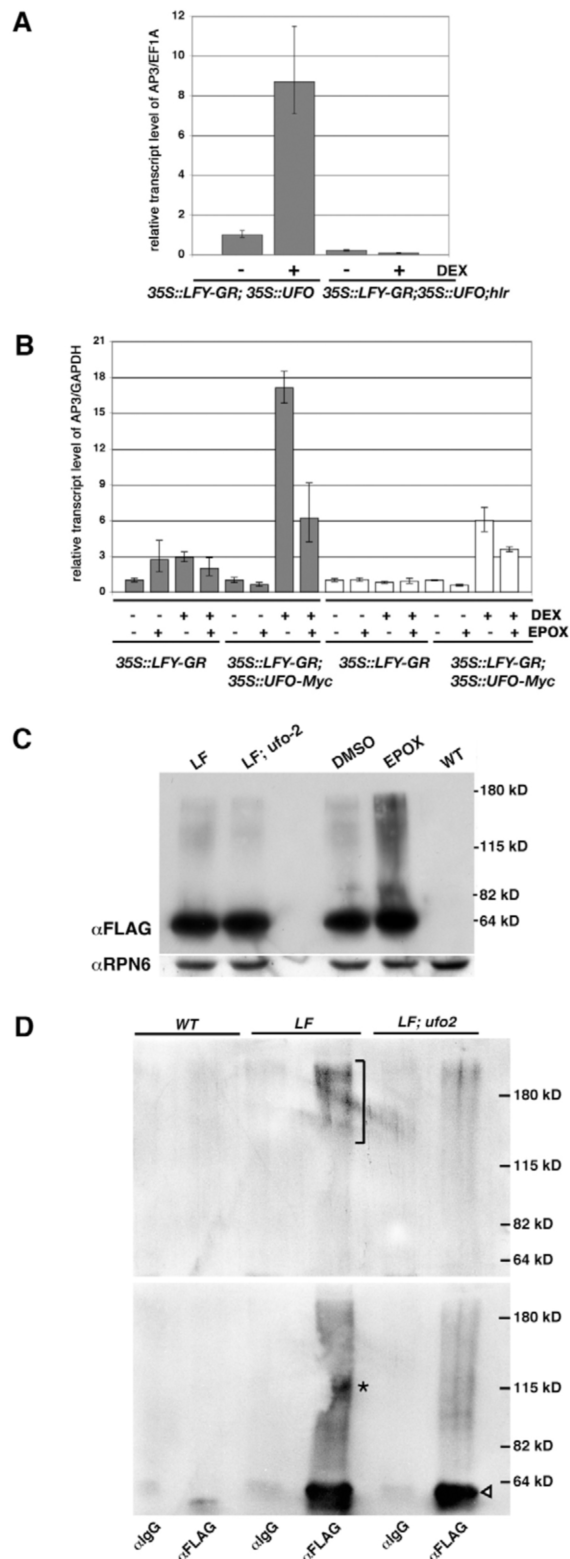

Fig. 6. Proteasome activity is required for the ectopic induction of *AP3* expression in seedlings expressing *35S::UFO* and *35S::LFY-GR*. (**A**) The *hlr* mutation abrogates DEX-dependent induction of *AP3* expression. Levels of *AP3* expression monitored by qRT-PCR and normalized to *EF-1α*. (**B**) Epoxomicin treatment reduces DEX induced *AP3* expression in seedlings that express *35S::UFO-Myc* and *35S::LFY-GR*. Gray and white bars represent two different biological replicates. Levels of *AP3* expression monitored by qRT-PCR and normalized to GAPDH. Ten plants assayed for each condition. Values represent mean±s.e.m. for the three technical replicates. (**C**) A subpopulation of LFY-FLAG protein is post-translationally modified and stabilized in the presence of epoxomicin. SDS-PAGE of LFY-FLAG protein as detected by αFLAG antibodies shows a prominent band of LFY-FLAG protein at approximately 55 kDa (lane 1), as well as a smear of higher molecular weight proteins. The levels of these higher molecular weight proteins are reduced in a *ufo-2* mutant background (lane 2). Conversely, an increase in the levels of these higher molecular weight proteins is seen in epoxomicin-treated 35S::LFY-FLAG inflorescences (lane 4) when compared with mock (DMSO)-treated tissue (lane 3). No crossreacting proteins are detected in wild-type samples (lane 5). Blot was reprobed with αRPN6 as a loading control. (**D**) LFY-FLAG protein is ubiquitylated. (Top panel) Immunoprecipitated LFY-FLAG protein or control IgG probed with anti-ubiquitin antibodies; (bottom panel) probed with anti-FLAG antibodies. Protein extracts from wild-type (WT), *35S::LFY-FLAG* (LF) or *35S::LFY-FLAG; ufo-2* (LF; ufo2) inflorescences. LFY-FLAG protein migrates at ~55 kDa (arrowhead), while polyubiquitylated species can be detected in the 150-220 kDa range (bracket). A ~115 kDa UFO-dependent band can be detected (asterisk) in the LF lane, suggesting LFY-FLAG is post-translationally modified in a UFO-dependent manner.

weight FLAG-tagged proteins are increased in epoxomicin-treated tissue when compared with mock DMSO treatment (Fig. 6C), suggesting that, in vivo, LFY is subject to ubiquitin-dependent proteasome mediated degradation. To confirm that the high molecular weight protein smear corresponds to ubiquitylated forms of LFY, or LFY-associated proteins, the immunoprecipitated LFY-FLAG protein, isolated from the inflorescences of *35S::LFY-FLAG* transgenic plants, was examined for the presence of ubiquitylated species in both *UFO* and *ufo-2* backgrounds (Fig. 6D). Polyubiquitylated species could be detected in immunoblots decorated with anti-ubiquitin antibody (Fig. 6D, upper panel). These observations indicate that LFY-FLAG, or tightly associated co-immunoprecipitated LFY-FLAG complex components, are polyubiquitylated in vivo. Furthermore, LFY-FLAG complex polyubiquitylation is partially abrogated in the *ufo-2* mutant background (Fig. 6D). This suggests that, in vivo, LFY (or LFY complex) ubiquitylation is partly dependent on UFO activity, but probably also depends on the action of other F-box proteins.

DISCUSSION

Nearly 700 F-box proteins are predicted to be encoded in the *Arabidopsis* genome (Gagne et al., 2002; Kuroda et al., 2002). The striking expansion of this gene family probably reflects the recruitment of F-box proteins to regulate a variety of biological processes, implicating protein degradation as a prevalent developmental control mechanism in plants. For example, the *Arabidopsis* F-box protein TIR1 mediates hormonal signaling by acting as an auxin receptor; auxin binding to TIR1 promotes the activity of SCF^{TIR1} in targeting Aux/IAA proteins for destruction (Dharmasiri et al., 2005; Kepinski and Leyser, 2005; Tan et al., 2007). Here, we describe a new role for a plant F-box protein, in functioning as a component of the transcriptional machinery that regulates floral homeotic gene expression.

The work presented here provides evidence that the F-box protein UFO functions as a transcriptional co-factor of LFY. We have shown that UFO physically interacts with LFY and that this interaction is

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necessary to recruit UFO to *AP3* promoter elements. Furthermore, UFO-Myc tagged protein is localized to the nucleus (E.C., K. Geuten and V.F.I., unpublished). These results imply that the physical interaction between LFY and UFO occurs in the context of the *AP3* promoter, and the abrogation of the association of UFO with the promoter in the absence of functional LFY strongly supports the idea. We also carried out transgenic studies in which a *UFO-SRDX* fusion construct was expressed ectopically, resulting in a *lfy*-like phenotype. These results indicate that converting UFO to a strong transcriptional repressor in vivo results in reduced LFY activity. Furthermore, we observed that LFY, or LFY-associated proteins, are ubiquitylated in vivo in a partially UFO-dependent manner, and that proteasome activity is required for *AP3* to be fully induced. Based on these observations, it is likely that SCF^{UFO} stimulates LFY-induced transcription via promoter-associated proteasome-mediated degradation of LFY, or of a factor in the LFY transcriptional complex.

Recently, several yeast and mammalian F-box proteins have been shown to promote the degradation of target transcription factors, but, surprisingly, coordinately promote their transcriptional activity (Kim et al., 2003; Muratani et al., 2005; Perissi et al., 2004; von der Lehr et al., 2003). However, the mechanism by which this transcriptional control is effected is still a matter of debate (Kodadek et al., 2006; Nalley et al., 2006). These observations, though, have led to the proposition that the rapid turnover of active transcriptional activators by the ubiquitylation-proteasome pathway, allowing for the removal of 'spent' activators, can explain how degradation of transcriptional activators can paradoxically stimulate target gene expression (Collins and Tansey, 2006; Kodadek et al., 2006; Lipford et al., 2005).

One possibility for how UFO functions to stimulate LFY transcriptional activity is through mediating polyubiquitylation of the LFY transcription factor (or tightly associated factors) at the *AP3* promoter, thus stimulating its rapid turnover and concomitant activation of *AP3* expression. To test this idea, we examined ubiquitylation in vivo, by using a FLAG-tagged version of LFY overexpressed in transgenic *Arabidopsis* plants (Fig. 6D). The ubiquitylated species detected by this method do not appear to be exclusively dependent on UFO, suggesting that LFY complex ubiquitylation depends on multiple E3 ligases, which could potentially modulate other LFY transcriptional activities. As LFY is involved in regulating the expression of multiple floral homeotic genes (Busch et al., 1999; Lamb et al., 2002; Lohmann et al., 2001; Parcy et al., 1998) as well as several meristem identity genes (Saddic et al., 2006; Wagner et al., 1999), it is conceivable that LFY transcriptional activity is differentially regulated by distinct interactions with different F-box proteins. Furthermore, overall LFY protein levels appear to be unaffected by UFO, which may reflect the possibility that UFO specifically acts to modulate the stability of promoter bound LFY.

Additionally, SCF^{UFO} may act to ubiquitylate other factors that are part of the transcriptional machinery at the *AP3* promoter; such factors would likely correspond to potential negative regulators of *AP3* transcription (Samach et al., 1999). Several negative regulators of *AP3*, including *SUPERMAN* (*SUP*), *EARLY BOLTING IN SHORT DAYS* (*EBS*) and the transcriptional co-repressor LEUNIG (LUG)/SEUSS (SEU) complex have been identified (Gomez-Mena et al., 2001; Sakai et al., 1995; Liu and Meyerowitz, 1995; Sridhar et al., 2004), but we have not been able to detect any interaction between UFO and those proteins (E.C. and V.F.I., unpublished; Z. Liu, personal communication), implying that they are not targets of SCF^{UFO} action. Thus, an as yet unidentified factor may play this role.

Mutant phenotypes for several eudicot *UFO* homologs suggest they all participate in a similar process of regulating *AP3* homolog gene expression, as they all disrupt petal and stamen development (Ingram, 1997; Taylor, 2001; Zhang, 2003; Ikeda et al., 2007). However, these mutations also result in ectopic flower production, a phenotype not seen in *Arabidopsis ufo* mutants. For example, Antirrhinum *fimbriata* mutants produce ectopic flowers in the axils of sepals (Ingram, 1997), similar to the phenotype produced by mutations in the Antirrhinum *AP1* homolog (Huijser, 1992). In legumes, mutations in the Pisum and Lotus *UFO* homologs produce indeterminate floral meristems resulting in extra floral organs (Taylor et al., 2001; Zhang et al., 2003), in a manner similar to that of mutations in the legume *AP1* homologs (Benlloch et al., 2006; Taylor et al., 2002). One possibility is that *UFO* is not only involved in regulating LFY-dependent transcription of *AP3*, but may also control the activity of the AP1 transcription factor in *Arabidopsis* (Fig. 5) (Hepworth et al., 2006). Together, these observations suggest that UFO may have a widely conserved role in regulating both LFY and AP1 transcriptional activities, as reflected in its mutant phenotypes in different species.

In summary, our results demonstrate that the UFO F-box protein acts as a transcriptional co-factor of LFY at the *AP3* promoter. These results also provide insights into how the activity of LFY, a master regulator of flowering, is tightly regulated to promote regional activation of downstream target floral homeotic genes, which in turn is necessary for specification and differentiation of individual floral organs.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/7/1235/DC1

References

Benlloch, R., d'Erfurth, I., Ferrandiz, C., Cosson, V., Beltran, J. P., Canas, L. A., Kondorosi, A., Madueno, F. and Ratet, P. (2006). Isolation of mtpim proves Tnt1 a useful reverse genetics tool in Medicago truncatula and uncovers new aspects of AP1-like functions in legumes. *Plant Physiol.* **142**, 972-983.

Blazquez, M. A. and Weigel, D. (2000). Integration of floral inductive signals in Arabidopsis. *Nature* **404**, 889-892.

Bowman, J. L., Alvarez, J., Weigel, D., Meyerowitz, E. M. and Smyth, D. R. (1993). Control of flower development in Arabidopsis thaliana by APETALA1 and interacting genes. *Development* **119**, 721-743.

Busch, M. A., Bomblies, K. and Weigel, D. (1999). Activation of a floral homeotic gene in Arabidopsis. *Science* **285**, 585-587.

Clough, S. J. and Bent, A. F. (1998). Floral dip: A simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. *Plant J.* **16**, 735-743.

Collins, G. A. and Tansey, W. P. (2006). The proteasome: a utility tool for transcription? *Curr. Opin. Genet. Dev.* **16**, 197-202.

Craig, K. L. and Tyers, M. (1999). The F-box: a new motif for ubiquitin dependent proteolysis in cell cycle regulation and signal transduction. *Prog. Biophys. Mol. Biol.* **72**, 299-328.

Deshaies, R. J. (1999). SCF and Cullin/Ring H2-based ubiquitin ligases. *Annu. Rev. Cell Dev. Biol.* **15**, 435-467.

Dharmasiri, N., Dharmasiri, S. and Estelle, M. (2005). The F-box protein TIR1 is an auxin receptor. *Nature* **435**, 441-445.

Feldman, R. M., Correll, C. C., Kaplan, K. B. and Deshaies, R. J. (1997). A complex of Cdc4p, Skp1p, and Cdc53p/cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1p. *Cell* **91**, 221-230.

Gagne, J. M., Downes, B. P., Shiu, S. H., Durski, A. M. and Vierstra, R. D. (2002). The F-box subunit of the SCF E3 complex is encoded by a diverse superfamily of genes in Arabidopsis. *Proc. Natl. Acad. Sci. USA* **99**, 11519-11524.

Gomez-Mena, C., Pineiro, M., Franco-Zorrilla, J. M., Salinas, J., Coupland, G. and Martinez-Zapater, J. M. (2001). Early bolting in short days: an Arabidopsis mutation that causes early flowering and partially suppresses the floral phenotype of leafy. *Plant Cell* **13**, 1011-1024.

Gustafson-Brown, C., Savidge, B. and Yanofsky, M. F. (1994). Regulation of the Arabidopsis homeotic gene APETALA1. *Cell* **76**, 131-143.
Hepworth, S. R., Klenz, J.E., and Haughn, G.W. (2006). UFO in the Arabidopsis inflorescence apex is required for floral-meristem identity and bract suppression. *Planta* **223,** 769-778.
Hill, T. A., Day, C. D., Zondlo, S. C., Thackeray, A. G. and Irish, V. F. (1998). Discrete spatial and temporal cis-acting elements regulate transcription of the Arabidopsis floral homeotic gene APETALA3. *Development* **125**, 1711-1721.
Hiratsu, K., Matsui, K., Koyama, T. and Ohme-Takagi, M. (2003). Dominant repression of target genes by chimeric repressors that include the EAR motif, a repression domain, in Arabidopsis. *Plant J.* **34**, 733-739.
Horwitz, A. A., Affar, el B., Heine, G. F., Shi, Y. and Parvin, J. D. (2007). A mechanism for transcriptional repression dependent on the BRCA1 E3 ubiquitin ligase. *Proc. Natl. Acad. Sci. USA* **104**, 6614-6619.
Huala, E. and Sussex, I. M. (1992). *LEAFY* interacts with floral homeotic genes to regulate *Arabidopsis* floral development. *Plant Cell* **4**, 901-913.
Ikeda, K., Ito, M., Nagasawa, N., Kyozuka, J. and Nagato, Y. (2007). Rice ABERRANT PANICLE ORGANIZATION 1, encoding an F-box protein, regulates meristem fate. *Plant J.* **51**, 1030-1040.
Ingram, G. C., Goodrich, J., Wilkinson, M. D., Simon, R., Haughn, G. W. and Coen, E. S. (1995). Parallels between UNUSUAL FLORAL ORGANS and FIMBRIATA, genes controlling flower development in Arabidopsis and Antirrhinum. *Plant Cell* **7**, 1501-1510.
Ingram, G. C., Doyle, S., Carpenter, R., Schultz, E. A., Simon, R. and Coen, E. S. (1997). Dual role for fimbriata in regulating floral homeotic genes and cell division in Antirrhinum. *EMBO J.* **16**, 6521-6534.
Ito, T., Takahashi, N., Shimura, Y. and Okada, K. (1997). A serine/threonine protein kinase gene isolated by an in vivo binding procedure using the Arabidopsis floral homeotic gene product, AGAMOUS. *Plant Cell Physiol.* **38**, 248-258.
Jack, T., Brockman, L. L. and Meyerowitz, E. M. (1992). The homeotic gene APETALA3 of Arabidopsis thaliana encodes a MADS box and is expressed in petals and stamens. *Cell* **68**, 683-697.
Kaiser, P., Flick, K., Wittenberg, C. and Reed, S. I. (2000). Regulation of transcription by ubiquitination without proteolysis: Cdc34/SCFMet30-mediated inactivation of the transcription factor Met4. *Cell* **102**, 303-314.
Kepinski, S. and Leyser, O. (2005). The Arabidopsis F-box protein TIR1 is an auxin receptor. *Nature* **435**, 446-451.
Kim, S. Y., Herbst, A., Tworkowski, K. A., Salghetti, S. E. and Tansey, W. P. (2003). Skp2 regulates Myc protein stability and activity. *Mol. Cell* **11**, 1177-1188.
Kodadek, T., Sikder, D. and Nalley, K. (2006). Keeping transcriptional activators under control. *Cell* **127**, 261-264.
Kubo, M., Udagawa, M., Nishikubo, N., Horiguchi, G., Yamaguchi, M., Ito, J., Mimura, T., Fukuda, H. and Demura, T. (2005). Transcription switches for protoxylem and metaxylem vessel formation. *Genes Dev.* **19**, 1855-1860.
Kuroda, H., Takahashi, N., Shimada, H., Seki, M., Shinozaki, K. and Matsui, M. (2002). Classification and expression analysis of Arabidopsis F-box-containing protein genes. *Plant Cell Physiol.* **43**, 1073-1085.
Lamb, R. S., Hill, T. A., Tan, Q. K. and Irish, V. F. (2002). Regulation of APETALA3 floral homeotic gene expression by meristem identity genes. *Development* **129**, 2079-2086.
Lee, I., Wolfe, D. S., Nilsson, O. and Weigel, D. (1997). A LEAFY co-regulator encoded by UNUSUAL FLORAL ORGANS. *Curr. Biol.* **7**, 95-104.
Lenhard, M., Bohnert, A., Jürgens, G. and Laux, T. (2001). Termination of stem cell maintenance in Arabidopsis floral meristems by interactions between WUSCHEL and AGAMOUS. *Cell* **105**, 805-814.
Levin, J. Z. and Meyerowitz, E. M. (1995). UFO: an Arabidopsis gene involved in both floral meristem and floral organ development. *Plant Cell* **7**, 529-548.
Lipford, J. R., Smith, G. T., Chi, Y. and Deshaies, R. J. (2005). A putative stimulatory role for activator turnover in gene expression. *Nature* **438**, 113-116.
Liu, Z. and Meyerowitz, E. M. (1995). LEUNIG regulates AGAMOUS expression in Arabidopsis flowers. *Development* **121**, 975-991.
Lohmann, J. U., Hong, R. L., Hobe, M., Busch, M. A., Parcy, F., Simon, R. and Weigel, D. (2001). A molecular link between stem cell regulation and floral patterning in Arabidopsis. *Cell* **105**, 793-803.
Long, J. A. and Barton, M. K. (1998). The development of apical embryonic pattern in Arabidopsis. *Development* **125**, 3027-3035.
Maizel, A., Busch, M. A., Tanahashi, T., Perkovic, J., Kato, M., Hasebe, M. and Weigel, D. (2005). The floral regulator LEAFY evolves by substitutions in the DNA binding domain. *Science* **308**, 260-263.
Meng, L., Mohan, R., Kwok, B. H., Elofsson, M., Sin, N. and Crews, C. M. (1999). Epoxomicin, a potent and selective proteasome inhibitor, exhibits in vivo antiinflammatory activity. *Proc. Natl. Acad. Sci. USA* **96**, 10403-10408.
Miller, S. L., Malotky, E. and O'Bryan, J. P. (2004). Analysis of the role of ubiquitin-interacting motifs in ubiquitin binding and ubiquitylation. *J. Biol. Chem.* **279**, 33528-33537.
Muratani, M. and Tansey, W. P. (2003). How the ubiquitin-proteasome system controls transcription. *Nat. Rev. Mol. Cell Biol.* **4**, 192-201.
Muratani, M., Kung, C., Shokat, K. M. and Tansey, W. P. (2005). The F box protein Dsg1/Mdm30 is a transcriptional coactivator that stimulates Gal4 turnover and cotranscriptional mRNA processing. *Cell* **120**, 887-899.
Nalley, K., Johnston, S. A. and Kodadek, T. (2006). Proteolytic turnover of the Gal4 transcription factor is not required for function in vivo. *Nature* **442**, 1054-1057.
Ni, W., Xie, D., Hobbie, L., Feng, B., Zhao, D., Akkara, J. and Ma, H. (2004). Regulation of flower development in Arabidopsis by SCF complexes. *Plant Physiol.* **134**, 1574-1585.
Nilsson, O., Lee, I., Blazquez, M. A. and Weigel, D. (1998). Flowering time genes modulate the response to LEAFY activity. *Genetics* **150**, 403-410.
Ostendorff, H. P., Peirano, R. I., Peters, M. A., Schluter, A., Bossenz, M., Scheffner, M. and Bach, I. (2002). Ubiquitination-dependent cofactor exchange on LIM homeodomain transcription factors. *Nature* **416**, 99-103.
Palombella, V. J., Rando, O. J., Goldberg, A. L. and Maniatis, T. (1994). The ubiquitin-proteasome pathway is required for processing the NF-kappa B1 precursor protein and the activation of NF-kappa B. *Cell* **78**, 773-785.
Parcy, F., Nilsson, O., Busch, M. A., Lee, I. and Weigel, D. (1998). A genetic framework for floral patterning. *Nature* **395**, 561-566.
Parcy, F., Bomblies, K. and Weigel, D. (2002). Interaction of LEAFY, AGAMOUS and TERMINAL FLOWER1 in maintaining floral meristem identity in Arabidopsis. *Development* **129**, 2519-2527.
Peirson, S. N., Butler, J. N. and Foster, R. G. (2003). Experimental validation of novel and conventional approaches to quantitative real-time PCR data analysis. *Nucleic Acids Res.* **31**, e73.
Perissi, V., Aggarwal, A., Glass, C. K., Rose, D. W. and Rosenfeld, M. G. (2004). A corepressor/coactivator exchange complex required for transcriptional activation by nuclear receptors and other regulated transcription factors. *Cell* **116**, 511-526.
Saddic, L. A., Huvermann, B., Bezhani, S., Su, Y., Winter, C. M., Kwon, C. S., Collum, R. P. and 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.
Sakai, H., Medrano, L. J. and Meyerowitz, E. M. (1995). Role of SUPERMAN in maintaining Arabidopsis floral whorl boundaries. *Nature* **378**, 199-201.
Samach, A., Klenz, J. E., Kohalmi, S. E., Risseeuw, E., Haughn, G. W. and Crosby, W. L. (1999). The UNUSUAL FLORAL ORGANS gene of Arabidopsis thaliana is an F-box protein required for normal patterning and growth in the floral meristem. *Plant J.* **20**, 433-445.
Schultz, E. A. and Haughn, G. W. (1991). LEAFY, a homeotic gene that regulates inflorescence development in Arabidopsis. *Plant Cell* **3**, 771-781.
Skowyra, D., Craig, K. L., Tyers, M., Elledge, S. J. and Harper, J. W. (1997). F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. *Cell* **91**, 209-219.
Sridhar, V. V., Surendrarao, A., Gonzalez, D., Conlan, R. S. and Liu, Z. (2004). Transcriptional repression of target genes by LEUNIG and SEUSS, two interacting regulatory proteins for Arabidopsis flower development. *Proc. Natl. Acad. Sci. USA* **101**, 11494-11499.
Tan, Q. K. and Irish, V. F. (2006). The Arabidopsis zinc finger-homeodomain genes encode proteins with unique biochemical properties that are coordinately expressed during floral development. *Plant Physiol.* **140**, 1095-1108.
Tan, X., Calderon-Villalobos, L. I., Sharon, M., Zheng, C., Robinson, C. V., Estelle, M. and Zheng, N. (2007). Mechanism of auxin perception by the TIR1 ubiquitin ligase. *Nature* **446**, 640-645.
Taylor, S., Hofer, J. and Murfet, I. I. (2001). Stamina pistilloida, the pea ortholog of Fim and UFO, is required for normal development of flowers, inflorescences, and leaves. *Plant Cell* **13**, 31-46.
Taylor, S. A., Hofer, J. M., Murfet, I. C., Sollinger, J. D., Singer, S. R., Knox, M. R. and Ellis, T. H. (2002). PROLIFERATING INFLORESCENCE MERISTEM, a MADS-box gene that regulates floral meristem identity in pea. *Plant Physiol.* **129**, 1150-1159.
Ueda, M., Matsui, K., Ishiguro, S., Sano, R., Wada, T., Paponov, I., Palme, K. and Okada, K. (2004). The HALTED ROOT gene encoding the 26S proteasome subunit RPT2a is essential for the maintenance of Arabidopsis meristems. *Development* **131**, 2101-2111.
von der Lehr, N., Johansson, S., Wu, S., Bahram, F., Castell, A., Cetinkaya, C., Hydbring, P., Weidung, I., Nakayama, K., Nakayama, K. I. et al. (2003). The F-box protein Skp2 participates in c-Myc proteosomal degradation and acts as a cofactor for c-Myc-regulated transcription. *Mol. Cell* **11**, 1189-1200.
Wagner, D., Sablowski, R. W. M. and Meyerowitz, E. M. (1999). Transcriptional activation of APETALA1 by LEAFY. *Science* **285**, 582-584.
Wang, H., Tang, W., Zhu, C. and Perry, S. E. (2002). A chromatin immunoprecipitation (ChIP) approach to isolate genes regulated by AGL15, a MADS domain protein that preferentially accumulates in embryos. *Plant J.* **32**, 831-843.
Wang, X., Feng, S., Nakayama, N., Crosby, W. L., Irish, V., Deng, X. W. and Wei, N. (2003). The COP9 Signalosome Interacts with SCF(UFO) and Participates in Arabidopsis Flower Development. *Plant Cell* **15**, 1071-1082.
Weigel, D. and Meyerowitz, E. M. (1993). Activation of floral homeotic genes in Arabidopsis. *Science* **261**, 1723-1726.
Weigel, D., Alvarez, J., Smyth, D. R., Yanofsky, M. F. and Meyerowitz, E. M. (1992). *LEAFY* controls floral meristem identity in *Arabidopsis*. *Cell* **69**, 843-859.

Wilkinson, M. D. and Haughn, G. W. (1995). UNUSUAL FLORAL ORGANS controls meristem identity and organ primordia fate in Arabidopsis. *Plant Cell* **7**, 1485-1499.
William, D. A., Su, Y., Smith, M. R., Lu, M., Baldwin, D. A. and Wagner, D. (2004). Genomic identification of direct target genes of LEAFY. *Proc. Natl. Acad. Sci. USA* **101**, 1775-1780.
Winston, J. T., Strack, P., Beer-Romero, P., Chu, C. Y., Elledge, S. J. and Harper, J. W. (1999). The SCFbeta-TRCP-ubiquitin ligase complex associates specifically with phosphorylated destruction motifs in IkappaBalpha and beta-catenin and stimulates IkappaBalpha ubiquitination in vitro. *Genes Dev.* **13**, 270-283.
Yaron, A., Gonen, H., Alkalay, I., Hatzubai, A., Jung, S., Beyth, S., Mercurio, F., Manning, A. M., Ciechanover, A. and Ben-Neriah, Y. (1997). Inhibition of NF-kappa-B cellular function via specific targeting of the I-kappa-B-ubiquitin ligase. *EMBO J.* **16**, 6486-6494.
Zhang, S., Sandal, N., Polowick, P. L., Stiller, J., Stougaard, J. and Fobert, P. R. (2003). Proliferating Floral Organs (Pfo), a Lotus japonicus gene required for specifying floral meristem determinacy and organ identity, encodes an F-box protein. *Plant J.* **33**, 607-619.
Zhao, D., Yang, M., Solava, J. and Ma, H. (1999). The ASK1 gene regulates development and interacts with the UFO gene to control floral organ identity in Arabidopsis. *Dev. Genet.* **25**, 209-223.

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