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Conservation versus divergence in *LEAFY* and *APETALA1* functions between *Arabidopsis thaliana* and *Cardamine hirsuta*

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Summary

- A conserved genetic toolkit underlies the development of diverse floral forms among angiosperms. However, the degree of conservation vs divergence in the configuration of these gene regulatory networks is less clear.
- We addressed this question in a parallel genetic study between the closely related species *Arabidopsis thaliana* and *Cardamine hirsuta*.
- We identified *leafy* (*lfy*) and *apetala1* (*ap1*) alleles in a mutant screen for floral regulators in *C. hirsuta*. *Cardamine hirsuta lfy* mutants showed a complete homeotic conversion of flowers to leafy shoots, mimicking *lfy ap1* double mutants in *A. thaliana*. Through genetic and molecular experiments, we showed that *AP1* activation is fully dependent on LFY in *C. hirsuta*, in contrast to *A. thaliana*. Additionally, we found that *LFY* influences heteroblasty in *C. hirsuta*, such that loss or gain of *LFY* function affects its

progression. Overexpression of *UNUSUAL FLORAL ORGANS* also alters *C. hirsuta* leaf shape in a LFY-dependent manner.

- We found that LFY and AP1 are conserved floral regulators that act nonredundantly in *C. hirsuta*, such that LFY has more obvious roles in floral and leaf development in *C. hirsuta* than *A. thaliana*.

Key words: *APETALA1*, *Cardamine hirsuta*, comparative development, leaf shape, *LEAFY*.

Introduction

Evo-devo studies seek to explain the developmental and genetic changes that shaped diversity. In plants, the astonishing diversity of angiosperm flowers provides an ideal system to address this question. Our current knowledge of the genetic control of flower development is based on initial work in two distantly related species: *Arabidopsis thaliana* and *Antirrhinum majus* (Coen & Meyerowitz, 1991). This comparison showed that conserved regulators specify the fate of floral meristems and floral organs in both species, despite their evolutionary distance and divergent flower morphology. Since then, there has been considerable interest in understanding how a common set of genes are reconfigured in species-specific regulatory networks to produce diverse floral forms.

Flower formation relies on the acquisition of floral meristem identity, conferred by the genes *LEAFY* (*LFY*) and *APETALA1* (*AP1*) in *A. thaliana* and the orthologous genes *FLORICAULA* (*FLO*) and *SQUAMOSA* (*SQUA*) in *A. majus* (Coen *et al.*, 1990; Irish & Sussex, 1990; Schwarz-Sommer *et al.*, 1990; Schultz & Haughn, 1991; Huala & Sussex, 1992; Mandel *et al.*, 1992; Weigel *et al.*, 1992; Shannon & Meeks-Wagner, 1993; Weigel & Meyerowitz, 1993). In *flo* mutants, flowers are homeotically converted to shoots because these meristems fail to acquire floral identity. The other three mutants, *lfy* and *ap1* in *A. thaliana* and *squa* in *A. majus*, have a similar phenotype although they show only a partial homeotic conversion. Specifically, the first flowers to initiate in a *lfy* mutant are converted into leafy shoots, but later flowers acquire partial floral identity (Schultz & Haughn, 1991; Weigel *et al.*, 1992). LFY is a transcription factor that directly activates the expression of various floral-organ identity genes, including the MADS-box gene *AP1* (Parcy *et al.*, 1998; Wagner *et al.*, 1999). Overexpression of *LFY* converts the inflorescence shoot

into a single terminal flower (Weigel & Nilsson, 1995). Therefore, *LFY* is sufficient and partially necessary for the acquisition of floral meristem identity in *A. thaliana*.

The development of flower-like structures in *lfy* mutants is caused by LFY-independent activation of *AP1* expression, since these flowers disappear when both *LFY* and *AP1* are mutated (Huala & Sussex, 1992; Weigel *et al.*, 1992; Weigel & Meyerowitz, 1993; Wagner *et al.*, 1999). In *lfy ap1* double mutants, flowers are homeotically converted to shoots, similar to *flo* single mutant flowers in *A. majus* (Coen *et al.*, 1990; Huala & Sussex, 1992; Weigel *et al.*, 1992). Therefore, *AP1* expression in *A. thaliana* is activated both in a LFY-dependent and -independent manner. For example, *AP1* expression is known to be directly induced by members of the SQUAMOSA BINDING PROTEIN-LIKE family, BLADE-ON-PETIOLE1 in concert with TGA transcription factors, the FLOWERING LOCUS D (FD) transcription factor together with FLOWERING LOCUS T (FT), the MADS-box proteins SHORT VEGETATIVE PHASE (SVP), AGAMOUS-LIKE24 (AGL24) and SEPALLATA3, and the transcription factor LATE MERISTEM IDENTITY2 (Wigge *et al.*, 2005; Kaufmann *et al.*, 2009; Yamaguchi *et al.*, 2009; Xu *et al.*, 2010; Pastore *et al.*, 2011; Grandi *et al.*, 2012). This raises the possibility that LFY-independent activation of *AP1* in *A. thaliana* may contribute to the milder consequences of *LFY* loss-of-function, in comparison to the homeotic phenotype of *flo* mutants in *A. majus*.

LFY interacts with the F-box protein UNUSUAL FLORAL ORGANS (UFO) in *A. thaliana* and this interaction is conserved among orthologs of these proteins in different flowering plants (Lee *et al.*, 1997; Chae *et al.*, 2008; Souer *et al.*, 2008). However, divergence in the spatiotemporal expression of these two genes played a major role in determining the various inflorescence architectures found in different species (Hake, 2008; McKim & Hay, 2010; Moyroud *et al.*, 2010; Park *et al.*, 2014; Kusters *et al.*, 2015). For example, *A. thaliana* and *A. majus* have a raceme architecture with lateral flowers, and *LFY/FLO* expression is the limiting factor for acquisition of floral fate in these flowers (Coen *et al.*, 1990; Blázquez *et al.*, 1997). *UFO* is expressed in both vegetative and reproductive tissues, and neither *UFO* or its *A. majus* ortholog *FIMBRIATA* are sufficient to specify floral meristem identity (Simon *et al.*, 1994; Lee *et al.*, 1997). In contrast to this, Solanaceae species such as petunia and tomato have a cyme architecture with terminal flowers, and rather than *LFY*, it is the *UFO* orthologs *DOUBLE TOP* and *ANANTHA* that are specifically expressed in these floral meristems and are necessary and sufficient to specify floral

identity (Souer *et al.*, 1998; Lippman *et al.*, 2008). Another example is *Gerbera hybrida*, where orthologs of *UFO* rather than *LFY* determine floral meristem identity in its capitulum inflorescence (Zhao *et al.*, 2016). Therefore, distinct inflorescence architectures were produced by variation in the gene expression patterns of conserved floral regulators.

In addition to flower development, *LFY* orthologs also regulate leaf development in some species. Particularly in legume species, such as *Pisum sativum* or *Medicago truncatula*, expression of the *LFY* orthologs *UNIFOLIATA* and *SINGLE LEAFLET1* is transiently activated in young leaves, and is required to produce a dissected leaf shape (Hofer *et al.*, 1997; Wang *et al.*, 2008; Chen *et al.*, 2010). However, this function for *LFY* is mostly restricted to a subclade of the Fabaceae (Champagne *et al.*, 2007). Throughout vascular plants, dissected leaf shape more commonly requires the co-option of genes active in the shoot apical meristem, such as class I *Knotted1-like homeobox* and *CUP-SHAPED COTYLEDON* genes, which pattern auxin maxima along the dissected leaf margin (Bharathan *et al.*, 2002; Hay & Tsiantis, 2006, 2010; Barkoulas *et al.*, 2008; Blein *et al.*, 2008; Koenig *et al.*, 2009). In the simple leaves of *A. thaliana*, overexpression of UFO changes the leaf margin from smooth to ruffled, and this requires *LFY* activity since these phenotypes disappear in a *lfy* background (Lee *et al.*, 1997; Chae *et al.*, 2008). Moreover, ectopic meristems form on leaves when UFO is fused with a VP16 transactivation domain in these experiments (Risseeuw *et al.*, 2013). These findings show that conserved floral regulators have evolved distinct functions in leaf development in some lineages.

In summary, current evidence suggests that functionally conserved orthologs of *LFY*, *AP1* and *UFO* contribute to floral initiation; and it is how these genes are wired in species-specific regulatory networks that is key to understanding floral diversity (Rosin & Kramer, 2009). In particular, it is important to understand whether LFY-independent activation of *AP1* involves relatively recent evolutionary events that are specific to the Arabidopsis lineage, rather than conserved features of angiosperm flower development. For example, the functions of *AP1* in sepal and petal development in *A. thaliana* may involve LFY-independent activation of *AP1* that is specific to this lineage (Ye *et al.*, 2016). Moreover, because LFY activity is required to produce a dissected leaf shape in some legume species (Hofer *et al.*, 1997; Wang *et al.*, 2008; Chen *et al.*, 2010), and also contributes to the development of dissected tomato leaves (Molinero-Rosales *et al.*, 1999), it is important to understand the

prevalence of this function of LFY. One approach to address these questions is to use parallel genetic studies in *A. thaliana* and its close relative *Cardamine hirsuta*. Both species belong to the Brassicaceae family, diverged *c*. 32 million yr ago (Mya) **[Author, please confirm inserted text 'million yr ago' is correct]**, and are reproductively isolated (Hay *et al.*, 2014). Comparative genetic analyses in these species have successfully identified molecular changes that underlie phenotypic differences that are of evolutionary significance, such as leaf shape and seed dispersal (Hay & Tsiantis, 2006; Barkoulas *et al.*, 2008; Vlad *et al.*, 2014; Hofhuis *et al.*, 2016; Vuolo *et al.*, 2016).

To determine the degree of conservation vs divergence in gene networks that control floral initiation in *A. thaliana* and *C. hirsuta*, we performed a genetic screen to identify *C. hirsuta* mutants with defects in floral meristem identity. Following this unbiased approach, we isolated alleles of *lfy* and *ap1* as important floral regulators in *C. hirsuta*. The *ap1* mutant phenotype was very similar between *C. hirsuta* and *A. thaliana*, however *C. hirsuta lfy* mutants showed a homeotic conversion of flowers to leafy shoots. We showed that this phenotype is explained by *AP1* expression being fully dependent on LFY activity in *C. hirsuta*. Moreover, we found that *LFY* was necessary for correct heteroblastic progression of leaf shape, and sufficient to alter this progression, in the dissected leaves of *C. hirsuta*. Finally, we showed that overexpression of *UFO* did not affect floral initiation, but increased the complexity of *C. hirsuta* leaves; and this required LFY activity. Our findings provide evidence of conserved and divergent functions of floral meristem identity genes between *A. thaliana* and *C. hirsuta*, and shed light on the evolution of *AP1* regulation.

Materials and Methods

Plant material and growth conditions

Cardamine hirsuta reference Oxford (Ox) accession, herbarium specimen voucher Hay 1 (OXF) (Hay & Tsiantis, 2006). The following *C. hirsuta* cDNA sequences have been deposited in GenBank: *ChLFY* (KX772396) and *ChAP1* (KX772395), and can also be found by these gene identifiers in the *C. hirsuta* genome assembly: *ChLFY* (CARHR275620) and *ChAP1* (CARHR062020) (Gan *et al.*, 2016). ABRC accession numbers for *A. thaliana* mutants used in this study are as follows: *lfy-6* (CS8552), *lfy-10* (CS6279), *ap1-12* (CS6232) and *ap1-1* (CS28). All plants were grown in long day conditions in the glasshouse: 16 h 22°C : 8 h 20°C, light : dark. For quantitative PCR

on seedling tissue, seeds were surface sterilized, stratified for 1 wk at 4°C and grown on 0.5 Murashige-Skoog medium for 8 d under long day conditions in a growth chamber. A *C. hirsuta lfy-2; ap1-119* double mutant was constructed by pollinating phenotypically wild-type individuals from a segregating *lfy-2* family with *ap1-119* pollen, selfing four *ap1-119* individuals in the F_2 generation, and identifying *lfy-2; ap1-119* double mutants segregating in the progeny of *ap1-119; lfy-2/+* parents.

Ethyl methyl sulfonate mutagenesis, mutant screening and cloning

Seeds (1500) of *C. hirsuta* Ox were washed with 0.1% Triton-X 100, agitated with 17 mM ethyl methyl sulfonate (EMS) for 10 h, washed 12 times with deionised H_2O, suspended in 0.1% agarose and sown on 1 : 1 soil : vermiculite mix. M_2 progeny were harvested as pools of five M_1 plants and 100 seeds each of 300 pools were sown and screened for defects in normal flower development.

Five alleles of *lfy* and three alleles of *ap1* were isolated. All mutants were backcrossed to Ox before further analysis. Molecular lesions and proof of cloning by transgenic complementation are described for alleles used in this study. The *lfy-2* sequence bares a G to A single nucleotide change at position 994 of the genomic sequence (starting from the ATG), predicted to convert a Try residue to a stop codon and produce a truncated 178 AA protein. The *lfy-3* sequence bears a C to T single nucleotide change at position 112 of the CDS **[Author, please insert expansion for 'CDS']**, predicted to convert a Gln residue to a stop codon and produce a truncated 37 AA protein. The *lfy*-4 sequence bears a C to T single nucleotide change at position 451 of the CDS, predicted to convert a Gln residue to a stop codon and produce a truncated 150 AA protein. The *lfy-3* mutant phenotype was complemented by expressing a *pAtLFY::AtLFY* transgene, described in the text, and other alleles were confirmed by allelism tests with *lfy-3*. The *ap1-119* sequence bears a G to A single nucleotide change at position 1855 of the genomic sequence (starting from the ATG), which modifies the splicing donor site of the second intron. The *ap1-797* sequence bears a G to A single nucleotide change at position 2592 of the genomic sequence, which modifies the splicing acceptor site of the fifth intron. Expressing a *gChAP1:GFP* translational fusion complemented the *ap1-119* mutant phenotype and other alleles were confirmed by allelism tests with *ap1-119*.

Transgenic plant construction

All binary vectors were transformed into *C. hirsuta* by *Agrobacterium tumefaciens* (strain GV3101) mediated floral dip.

35S::AtLFY was constructed in the destination vector pB2GW7 by recombination with the *AtLFY* cDNA in pENTR221 (DQ447103, ABRC). Forty independent lines were generated in both segregating *C. hirsuta lfy-3* and *A. thaliana lfy-6* backgrounds. T_3 lines homozygous for the transgene were identified in homozygous mutant and wild-type backgrounds. Plants were genotyped for the *lfy-3* mutation using the primer pair *lfy3_RsaI-1F* (5'- CCTGAAGGTTTCACGAGTGGC) and *lfy3_int1-R* (5'- TGACAAGTGTTGTTGGGAAG), producing a 614-bp amplicon digested by AccI into 108-bp and 506-bp fragments in the mutant allele. Plants were genotyped for the *lfy-6* mutation using the primer pair *lfy-6_Mae3-F* (5'- TATGGATCCTGAAGGTTTCACG) and *lfy-6_Mae3-R* (5'- CGGGCATAGAAATGTTG) (www.weigelworld.org).

Forty independent lines of *pAtLFY::AtLFY* (pETH29) (Chahtane *et al.*, 2013) were generated in a segregating *C. hirsuta lfy-3* background and a T_3 line homozygous for both the transgene and the *C. hirsuta lfy-3* allele was used for further analysis. This line was confirmed by seed fluorescence (Bensmihen *et al.*, 2004) and by genotyping with the primer pair *lfy3_RsaI-1F* and *lfy3_int1-R*.

For *35S::AtAP1* and *35S::ChAP1* constructs, the *AtAP1* cDNA was subcloned from pUNI51 (U20604, ABRC) into pBluescript SK and the *ChAP1* cDNA was amplified from *C. hirsuta* cDNA synthesized from RNA extracted from floral apices and cloned in pCRBlunt. *AtAP1* and *ChAP1* cDNAs were subcloned behind the CaMV *35S* promoter of the pART7 vector and the *35S::AtAP1* and *35S::ChAP1* cassettes were transferred to the binary vector pMLBART. Forty independent lines were generated for each construct in *A. thaliana ap1-1* and *ap1-12* and a subset were analysed in the T_2 generation.

gChAP1:GFP was constructed in the destination vector pMDC107 by recombination of a 6.6 kb genomic *C. hirsuta AP1* fragment in pCR8, which was generated by PCR amplification from a BAC containing the *C. hirsuta AP1* locus (SIU_BAC 20-M1) with the primers *ChAP1pro-F* (5'- CGTGGTGGTTAGAAGATAGCGTCAAC) and *ChAP1cterm-R* (5'- TGCGGCGAAGCAGCCAAGGTT). Ten independent lines of *gChAP1:GFP* were generated in *C. hirsuta ap1-119*.

The *35S:UFOi* plasmid (pJP61a) was a gift from P. Laufs (Laufs *et al.*, 2003) and independent insertion lines were generated in *C. hirsuta* wild type plants. Ethanol induction was performed as previously described (Deveaux *et al.*, 2003).

Quantitative RT-PCR analysis

Rosette leaves and whole inflorescences from *C. hirsuta* wild-type adult plants were used to measure *LFY* expression levels. Whole 8-d-old seedlings of *C. hirsuta* wild-type and *35S::AtLFY* plants were used to measure *LFY* and *AP1* expression levels. These *35S::AtLFY* plants were segregating for the *lfy-3* allele. Total RNA was extracted from three biological replicates of each tissue using the Spectrum Plant Total RNA Kit (Sigma-Aldrich). RNA was converted into cDNA using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific) and an oligo-dT primer. Quantitative PCR was performed in triplicate using Power SYBR Green Master Mix (Thermo Fisher Scientific) and the ViiA 7 Real-Time PCR System (Thermo Fisher Scientific). Primer efficiency and expression level were determined as previously described (Pfaffl, 2001). Expression levels of *LFY* (5'-CCAAGAAGGCTTATCAGAGGAGCCG-3' and 5'-CCGTCTTTGCTGTTGCTTCTTCATCT-3') and *AP1* (5'-TGGGTGGTCTGTATCAAGAAGAAG-3' and 5'-TATATGGAAATGCTTCATGCGGC-3') were normalized to the reference gene *CLATHRIN/AP2M* (5'-TCGATTGCTTGGTTTGGAAGATAAGA-3' and 5'-TTCTCTCCCATTGTTGAGATCAACTC-3').

Sequence analysis

Amino acid sequences for ChAP1 and ChLFY were derived from *in silico* translation of cDNA sequences amplified from *C. hirsuta* cDNA synthesized from RNA extracted from floral apices. The ChAP1 and ChLFY protein sequences were aligned to AtAP1 and AtLFY, respectively, using the MUSCLE (MUltiple Sequence Comparison by Log- Expectation) tool available online (http://www.ebi.ac.uk/Tools/mafft/index.html) using the BLOSUM62 matrix and percent identity calculated by pairwise alignment in Jalview. The alignment residues were colour-coded based on identity and conservation using AMAS server (http://www.compbio.dundee.ac.uk/www-amas). LFY binding sites were predicted in *A. thaliana* and *C. hirsuta AP1* regulatory regions as previously described (Moyroud

et al., 2011). A score is computed on a 19-bp fragment and is negatively proportional to the *in vitro* affinity of LFY for the fragment (Moyroud *et al.*, 2011).

In situ hybridisation

Shoot apices were induced to flower by a shift from short to long day conditions. For *in situ* hybridisation, apices were fixed in 4% paraformaldehyde, processed through to paraffin using a Tissue-Tek® processor (Sakura Finetek USA, Inc.) and 8 μm sections were hybridised with *C. hirsuta LFY* and *AP1* RNA probes as previously described (Hay & Tsiantis, 2006). Probes were amplified from *C. hirsuta* cDNA synthesised from RNA extracted from floral apices to give the following fragments: *ChLFY*, 1263-bp; *ChAP1*, 1400-bp.

Scanning electron microscopy

Shoot apices were induced to flower by a shift from short to long day conditions and fixed in FAA **[Author, please insert expansion for 'FAA' (if appropriate)]**, post-fixed in osmium tetraoxide, dehydrated, critical point dried and dissected before coating with gold/palladium for viewing in a JSM-5510 microscope (JEOL).

Leaf shape analysis

Shape variation in the terminal leaflets of *C. hirsuta* genotypes was quantified using Extended Eigenshape analysis as previously described (MacLeod, 1999; Cartolano *et al.*, 2015). Leaves of *A. thaliana* genotypes were adhered to white paper using spray adhesive and digitally scanned. Images were converted into binary images, and leaf area and perimeter were automatically computed using the ImageJ plugin IJBlob (Wagner & Lipinski, 2013). The leaf dissection index was calculated as perimeter squared / 4Π × area (Bai *et al.*, 2010).

Results

Cardamine hirsuta lfy mutants show homeotic conversion of flowers to leafy shoots

To identify floral regulators in *C. hirsuta*, we screened an EMS-mutagenised *C. hirsuta* population for floral meristem identity defects and isolated five *lfy* mutants (Fig. 1a–h). Sequencing of three alleles, *lfy-2*, *lfy-3* and *lfy-4*, revealed single nucleotide polymorphisms (SNPs) in the *C. hirsuta LFY* CDS, generating premature stop codons predicted to produce truncated 177 AA, 37 AA and 150 AA proteins,

respectively (Fig. 1h). We complemented the *lfy-3* mutant phenotype with an *A. thaliana LFY* transgene (*pAtLFY::AtLFY*; Fig. 2f–l). We confirmed that all other alleles belonged to a single complementation group by allelism tests with *lfy-3*.

We exploited this allelic series of *lfy* mutants in *C. hirsuta* to assess the degree of conservation in *LFY* gene function by comparison with *lfy* alleles in *A. thaliana*. We detected a striking difference in *lfy* phenotypes between species: all *lfy* alleles in *C. hirsuta* lacked floral meristem identity and instead formed a continuous phyllotactic spiral of leaves in the axils of bracts, which are cryptic in wild-type flowers (Fig. 1c–g). This indicates a complete homeotic flower-to-inflorescence conversion in these mutants. By contrast, even the null *lfy-6* allele in *A. thaliana* showed only partial homeotic conversion, producing flowers subtended by a bract that retain multiple floral features including whorled phyllotaxy, sepals and central carpels that are fused or unfused (Fig. 1i–l) (Schultz & Haughn, 1991; Weigel *et al.*, 1992). Complete conversion of flowers to leafy shoots is only observed in *A. thaliana* when both *LFY* and *AP1* function is lost (Fig. 1m–p) (Huala & Sussex, 1992; Weigel *et al.*, 1992; Weigel & Meyerowitz, 1993; Wagner *et al.*, 1999). Therefore, *lfy* single mutants in *C. hirsuta* phenocopy *lfy ap1* double mutants in *A. thaliana.*

The bracts subtending leafy shoots in *C. hirsuta lfy* mutants have a dissected shape, similar to cauline leaves of wild-type *C. hirsuta*, while bracts in *A. thaliana lfy* resemble the simple cauline leaf shape found in wild-type *A. thaliana* (Fig. 1c,k). Cauline leaves were continuously produced along the stem of all *C. hirsuta lfy* alleles, compared with the production of only three to four cauline leaves in wild type (Fig. 1g). The small leaves produced in the leafy shoots of *C. hirsuta lfy* are also dissected, unlike wild-type sepals, which are simple (Fig. 1b,d). Therefore, the shape of lateral organs produced by the inflorescence of *lfy* mutants in *C. hirsuta* vs *A. thaliana* differs for two reasons: first, because of a difference in leaf bauplan between species and second, because sepals are produced in the flower-like structures in *A. thaliana* but not *C. hirsuta lfy* mutants.

LFY function is conserved between *A. thaliana* and *C. hirsuta*

We hypothesised that the divergence in *lfy* phenotypes between *C. hirsuta* and *A. thaliana* reflected species-specific differences in either *LFY* or *AP1* function and sought to discriminate between these two possibilities. To start with, we examined whether *LFY* gene expression or function differed between *A. thaliana* and *C. hirsuta*

and found several lines of evidence to suggest conservation rather than divergence. First, we found that *C. hirsuta LFY* (*ChLFY*) expression was significantly upregulated in inflorescence vs leaf tissue, and strongly expressed in floral meristems initiating at the flanks of the inflorescence meristem, a similar pattern to that observed in *A. thaliana* (Fig. 2a,b) (Weigel *et al.*, 1992). Second, we showed that overexpressing the *A. thaliana LFY* cDNA from the CaMV *35S* promoter in either *A. thaliana* or *C. hirsuta* led to a comparable acceleration of flowering and conversion of axillary shoots to terminal flowers (Fig. 2c,d), suggesting that *A. thaliana LFY* is sufficient to cause flowering and ectopic flower formation in either species (Weigel & Nilsson, 1995). We also found that *AP1* expression was significantly upregulated in 8-d-old *C. hirsuta 35S::AtLFY* seedlings (Fig. 2e), suggesting that LFY activates *AP1* expression in *C. hirsuta* similarly to *A. thaliana* (Parcy *et al.*, 1998; Wagner *et al.*, 1999). Consistent with this result, we showed that the best LFY binding site in the *A. thaliana AP1* promoter is likely conserved in the *AP1* promoter of *C. hirsuta*, and is predicted to have a high affinity for LFY in both species (see later Supporting Information Fig. S6) (Benlloch *et al.*, 2011; Moyroud *et al.*, 2011; Winter *et al.*, 2011). Finally, we tested whether expression of *A. thaliana LFY* from its own promoter (*pAtLFY::AtLFY*) complemented the *lfy* phenotype in *C. hirsuta*. We found that transformants recovered wild-type flower and floral organ production in *C. hirsuta lfy-3*, in the same manner as in *A. thaliana lfy* (Fig. 2f–l) (Blázquez *et al.*, 1997). Therefore, *LFY* gene expression and function seem to be conserved between species, and LFY proteins from each species share 94% amino acid sequence identity (Fig. S1), suggesting that this is not the cause of the divergent *lfy* phenotype between *C. hirsuta* and *A. thaliana*.

Species-specific differences in *AP1* regulation

Next, we examined whether differences in *AP1* gene expression or function might explain the homeotic *lfy* phenotype in *C. hirsuta*. *Cardamine hirsuta AP1* (*ChAP1*) is expressed in floral meristems initiating at the flanks of the inflorescence meristem in a similar domain to *ChLFY* (Fig. 3a). *Arabidopsis thaliana AP1* (*AtAP1*) shares this wild-type expression pattern and is also expressed in *lfy* mutants due to activation by additional floral regulators, although the onset of expression is slightly delayed as compared with wild type plants (Liljegren *et al.*, 1999; Wagner *et al.*, 1999). Surprisingly, we did not detect any *ChAP1* expression in *C. hirsuta lfy-3* by *in situ*

hybridisation (Fig. 3b). To maximise our chances of detecting *AP1* expression we performed these experiments with samples collected >2 wk after floral induction by which time *AP1* expression was easily detected in multiple *A. thaliana lfy* alleles (Liljegren *et al.*, 1999; Wagner *et al.*, 1999). Thus, *AP1* expression in *C. hirsuta* appears entirely dependent upon LFY activity, in striking contrast to *AP1* expression in *A. thaliana*.

To investigate *ChAP1* function, we isolated two *ap1* alleles from an EMS-mutagenised *C. hirsuta* population, *ap1-119* and *ap1-797*, which showed a characteristic phenotype of branched flowers and petal loss (Figs 3c–j, S2a,b). Sequencing these *ap1* alleles revealed a SNP that mutates the splice donor site of the second intron in *ap1-119*, and the splice acceptor site of the fifth intron in *ap1-797* (Fig. 3d). We complemented the *ap1-119* mutant phenotype with a *C. hirsuta* AP1:GFP translational fusion (*gChAP1:GFP*, Fig. S2c) and crossed the *ap1-797* allele with *ap1-119* to confirm allelism. The branched flowers found in *C. hirsuta ap1-119* are due to ectopic floral meristems formed in the axils of first-whorl floral organs that reflect a partial transformation of sepals into leaves with associated axillary meristems (Fig. 3f,g). Floral organ development is also altered, particularly in the first two whorls. For example, sepals are flanked by stipules, which normally form at the base of leaves, and lateral sepals initiate lower on the receptacle and often abort (Fig. 3h). Comparable defects are found in *A. thaliana ap1* mutants, indicating a conserved function for *AP1* in regulating floral meristem identity and sepal and petal development in these species (Irish & Sussex, 1990; Bowman *et al.*, 1993). To further compare the function of *A. thaliana AP1* and *C. hirsuta AP1*, we used the CaMV *35S* promoter to overexpress the CDS of each gene in *A. thaliana ap1* mutants. Transformants expressing either construct showed equivalent acceleration of flowering, conversion of axillary shoots to terminal flowers and rescue of branching and petal loss in flowers (Fig. S2d–i; Table S1) (Mandel & Yanofsky, 1995). These results, together with 97% amino acid identity shared between *C. hirsuta* and *A. thaliana* AP1 (Fig. S3), support the conclusion that *AP1* function is conserved between species.

We used genetics to explore the functional significance of our observation that the inflorescence of *C. hirsuta lfy* mutants lacked *AP1* expression. If *AP1* activation is completely dependent on *LFY* in *C. hirsuta*, then we predicted that *lfy* mutants would show complete epistasis to *ap1*. We tested this prediction by constructing *lfy ap1*

double mutants and found that these double mutants were indistinguishable from single *lfy* mutants in *C. hirsuta* (Fig. 3i–l). Therefore, the genetic interaction between *LFY* and *AP1* differs between species. The additive interaction in *A. thaliana* (Fig. 1i–p) reflects both *LFY*-dependent and *LFY*-independent activation of *AP1*, while the epistatic interaction in *C. hirsuta* (Fig. 3i–l) is likely to reflect only *LFY*-dependent activation of *AP1*.

To directly test whether this species-specific difference in *AP1* regulation was responsible for phenotypic differences between *lfy* mutants in *A. thaliana* vs *C. hirsuta*, we overexpressed *AP1* in the *C. hirsuta lfy* mutant. We predicted that the *lfy* mutant would no longer have a homeotic phenotype in *C. hirsuta* if *AP1* was expressed. We found that the *35S::AtAP1* transgene was sufficient to recover floral organ identity in *C. hirsuta lfy-2*, such that flowers comprised sepals and central unfused carpels (Fig. 3m–p); essentially converting *C. hirsuta lfy* to an *A. thaliana lfy* phenotype. Taken together, our findings show that species-specific differences in *AP1* expression underlie the difference in *lfy* phenotypes between *A. thaliana* and *C. hirsuta*.

LFY regulates heteroblastic leaf shape in *C. hirsuta*

A role for *LFY* orthologs in determining leaf shape has been shown in a number of species with dissected leaves, particularly legumes in a subclade of the Fabaceae (Hofer *et al.*, 1997; Champagne *et al.*, 2007; Wang *et al.*, 2008; Chen *et al.*, 2010). We took advantage of *C. hirsuta lfy* mutants to assess the contribution of *LFY* to dissected leaf shape in a species in the Brassicaceae family. The shape of successive leaves differs in many plants, including *C. hirsuta*, in an age-dependent process called heteroblasty, tracking progressive phases of plant life from juvenile to adult, and vegetative to reproductive (Telfer *et al.*, 1997; Cartolano *et al.*, 2015). In *C. hirsuta*, leaf shape changes during aging by increasing leaflet number and altering leaflet shape from kidney- to wedge-shape, which is particularly pronounced in terminal leaflets (Fig. 4a) (Cartolano *et al.*, 2015). We found that this heteroblastic progression was delayed in *lfy-3* mutants such that leaves had significantly fewer leaflets than wild type from leaf 3 onwards, and failed to produce the maximum number of leaflets found in wild-type adult leaves (Fig. 4a,b). This heteroblastic delay was not associated with a significant delay in *lfy-3* flowering time, as both mutant and wild type produced a similar number of rosette leaves before flowering (Figs 4c, S4a).

We quantified terminal leaflet shape by Extended Eigenshape analysis, a multivariate approach based on outline analysis (MacLeod, 1999; Cartolano *et al.*, 2015). We found that the first principal component eigenvalue (ES1) accounts for 10.3% of the total shape variation found between the terminal leaflets of all genotypes, and quantifies the transition in shape from a juvenile kidney-shape to an adult wedge-shape (Figs 4d, S4b). Again, we found that heteroblastic progression was delayed in *lfy-3* mutants such that terminal leaflets had significantly lower ES1 eigenscore values than wild type from leaf 8 onwards, and failed to acquire the maximum ES1 value found in wild-type adult leaves (Fig. 4d). In contrast to this, we found significantly higher ES1 values in terminal leaflets of *35S::AtLFY* transgenic lines in *C. hirsuta* than wild type, from leaf 5 onwards, and precocious acquisition of the maximum ES1 value found in wild type (Fig. 4d). Flowering was also accelerated in *35S::AtLFY*, such that fewer rosette leaves were formed, and the maximum number of leaflets found in wild-type adult leaves was never reached on *35S::AtLFY* leaves before flowering (Fig. 4b,c). Our findings indicate that *LFY* provides a key input into the heteroblastic progression of *C. hirsuta* leaf shape and that altering its activity is both necessary and sufficient to alter this progression. Loss of *LFY* function reduces the rate of shape change in terminal leaflets, such that adult shape is never reached, while *LFY* overexpression accelerates this change, such that adult shape is reached precociously. Given that leaflet number is reduced in *35S::AtLFY*, compared with wild type, *LFY* overexpression may also disrupt other aspects of leaf development in addition to heteroblasty. However, the heteroblastic effect of *LFY* is obvious when simply comparing the terminal leaflet shape of the last rosette leaf before flowering between these *C. hirsuta* genotypes (indicated in Fig. 4a). In contrast to this, we detected no difference in the shape of the last rosette leaf between wild type and *lfy* mutants in *A. thaliana* (Figs 4e, S4c). Therefore, the contribution of *LFY* activity to heteroblastic leaf shape variation is more pronounced in *C. hirsuta* than *A. thaliana*.

LFY is required for *UFO* function in *C. hirsuta*

Since *UFO* overexpression alters leaf shape in a *LFY*-dependent manner in *A. thaliana* (Lee *et al.*, 1997; Chae *et al.*, 2008; Risseeuw *et al.*, 2013), we tested whether this function was conserved in *C. hirsuta*. We found that, similar to *A. thaliana*, expressing an ethanol-inducible version of *UFO* (*UFOi*) broadly under the *35S* promoter (Laufs *et al.*, 2003) alters the dissected leaf shape of *C. hirsuta* by

increasing its complexity (Figs 5a–d, S5a–c). This phenotype was dependent on LFY activity since the supernumerary leaflets and lobes disappeared in *35S::UFOi lfy-2* plants (Fig. 5e,f). Moreover, overexpression of *UFO* did not accelerate flowering in *C. hirsuta* (Figs 5g, S5d), suggesting that LFY is the limiting factor for floral initiation in both *C. hirsuta* and *A. thaliana*. These results suggest that LFY and UFO functions are potentially conserved between *C. hirsuta* and *A. thaliana*, although future work will help to determine the precise role of *UFO* in *C. hirsuta* development.

Discussion

Floral initiation is a critical point in a plant's life. In *C. hirsuta*, we found this irreversible switch to floral development is specified by the concerted action of *LFY* and *AP1*, similar to *A. thaliana*. However, in contrast to *A. thaliana*, the activation of *AP1* expression is entirely dependent on LFY in *C. hirsuta*. As a consequence, flowers are homeotically converted to shoots with cauline leaves in *C. hirsuta lfy* mutants, because these meristems fail to acquire floral identity. This is in stark contrast to *A. thaliana*, where LFY-independent activation of *AP1* maintains the development of flower-like structures in *lfy* mutants. We uncovered an additional function for *LFY* as necessary and sufficient for the heteroblastic progression of dissected leaf shape in *C. hirsuta*. Leaf shape is also modified by *UFO* overexpression, which markedly increased the complexity of *C. hirsuta* leaves; and like *A. thaliana*, it requires LFY activity for this function. Our findings show that *LFY*, *AP1*, and most likely *UFO*, are functionally conserved floral regulators in *C. hirsuta*. However, LFY has more obvious roles in the floral and leaf development of *C. hirsuta* than *A. thaliana*. This difference arises from differential *AP1* regulation during floral development, and divergent gene regulatory networks operating in simple vs dissected leaf development.

Divergent *AP1* regulation between *A. thaliana* and *C. hirsuta*

In *A. thaliana*, *LFY* and *AP1* act in a partially redundant manner to determine the identity of the floral meristem. This is not the case in *C. hirsuta*. Three independent *lfy* alleles show complete loss of floral meristem identity in *C. hirsuta*. The position and nonsense nature of the mutations, and the fact that all three alleles showed an identical phenotype, suggests that these are null alleles. Our results show that *LFY* acts nonredundantly to specify floral identity in *C. hirsuta* because *AP1* activation is

completely dependent on LFY. This suggests that components responsible for LFY-independent induction of *AP1* may have diverged between *A. thaliana* and *C. hirsuta*. LFY-independent activation of *AP1* is thought to be achieved by the FT–FD complex, since double mutants between *lfy ft* and *lfy fd* mimic the homeotic phenotype of *lfy ap1* double mutants (Wigge *et al.*, 2005). However, the exact *cis*-element that FT–FD binds in the *AP1* promoter is still unknown (Benlloch *et al.*, 2011). A recently evolved MADS-box transcription factor binding site (CArG box) was identified in the *AP1* promoter of *A. thaliana*, via which CAULIFLOWER and AP1 itself could induce *AP1* expression (Ye *et al.*, 2016). SVP and AGL24 are additional MADS-box proteins that could activate *AP1* via this CArG box, independent of LFY, since double mutants between *lfy svp* and *lfy agl24* also mimic the phenotype of *lfy ap1* double mutants (Grandi *et al.*, 2012). In comparison to the *A. thaliana* CArG box sequence, there are two mutations and one deletion in the *C. hirsuta* sequence, suggesting it is nonfunctional, and a possible candidate to explain why regulation of *AP1* in *C. hirsuta* is completely dependent on LFY (Fig. S6). Despite this difference, our analysis of *C. hirsuta ap1* mutants shows that *AP1* is required for sepal and petal development in both *C. hirsuta* and *A. thaliana* and that this is not a derived function of *AP1* in *A. thaliana* (Ye *et al.*, 2016). Future work will help to identify the precise regulatory changes that underlie the difference in *AP1* regulation between *A. thaliana* and *C. hirsuta*.

Previous studies have reported both partial and full homeotic conversions of flowers to shoots in orthologous *lfy* mutants in various flowering plants (Coen *et al.*, 1990; Weigel *et al.*, 1992; Hofer *et al.*, 1997; Molinero-Rosales *et al.*, 1999; Bomblies *et al.*, 2003; Dong *et al.*, 2005; Souer *et al.*, 2008; Wang *et al.*, 2008; Ikeda-Kawakatsu *et al.*, 2012; Zhao *et al.*, 2016). This suggests that the relative role of *LFY* vs other regulators of floral meristem identity is evolutionary labile. It will be interesting to understand whether differences in *AP1* regulation underlie not only the difference between *A. thaliana* and *C. hirsuta lfy* phenotypes, but have evolved repeatedly, and contribute to the variable floral phenotypes of *lfy* mutants across angiosperms. Generating additional mutants in *LFY* orthologs in other species, particularly in *A. thaliana* relatives, should help resolve this question.

LFY influences the heteroblastic progression of leaf shape in *C. hirsuta*

Previous work showed that regulatory divergence in *FLOWERING LOCUS C* (*FLC*) underlies much of the natural variation in *C. hirsuta* leaf shape (Cartolano *et al.*, 2015). Low-expressing *FLC* alleles accelerate both flowering time and heteroblastic progression of leaf shape, resulting in a faster progression to adult leaf shape. This work showed that *FLC* coordinates leaf development with reproductive timing, and that this coordination influences seed yield (Cartolano *et al.*, 2015). Here we found that *LFY* also influences the heteroblastic progression of *C. hirsuta* leaf shape, such that *LFY* is required to produce an adult leaf shape. However, we observed no flowering time delay in the *C. hirsuta lfy* mutant. This finding suggests that the role of *LFY* in heteroblasty may be independent of the floral transition. There are at least two possible explanations for this: first, the low level of *LFY* expression in leaves (Fig. 2b) may promote adult traits or second, LFY-dependent signals that are produced after bolting may feedback to influence leaf development. This latter possibility is consistent with the work on *FLC* (Cartolano *et al.*, 2015), which suggests that the transition to flowering is accompanied by developmental changes in leaves that prepare the plant for impending reproduction.

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Author contributions

A.H. designed and directed the project; A.H., M.M., S.M.M. and M.C. performed research; F.P. and E.T. contributed materials and M.T. contributed to leaf shape

analysis. A.H. wrote the paper with help from M.M. and S.M.M. **[Author, please note amended initials S.M.M. (to match author list).]**

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Supporting Information

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

Fig. S1 Alignment of LFY proteins from *Arabidopsis thaliana* and *Cardamine hirsute.*

Fig. S2 AP1 protein function is conserved between *Arabidopsis thaliana* and *Cardamine hirsute.*

Fig. S3 Alignment of AP1 proteins from *Arabidopsis thaliana* and *Cardamine hirsute.*

Fig. S4 LFY does not influence *Cardamine hirsuta* flowering time or *Arabidopsis thaliana* leaf shape.

Fig. S5 Induction of *UFO* overexpression affects *Cardamine hirsuta* leaf shape but not flowering time.

Fig. S6 Comparative analysis of LFY binding sites and a CArG box between the *AP1* regulatory regions of *Arabidopsis thaliana* vs *Cardamine hirsute.*

Table S1 Organ counts for *35S::AP1* genotypes

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Fig. 1 *Cardamine hirsuta lfy* mutants resemble *lfy ap1* double mutants of *Arabidopsis thaliana*. (a, b) *Cardamine hirsuta* wild-type (a) inflorescence and (b) flower with floral organs arranged in whorls. (c, d) *Cardamine hirsuta lfy-3* inflorescence shows (c) complete floral to shoot conversion with (d) flowers consisting of leaves in a spiral arrangement. (e, f) *Cardamine hirsute* (e) *lfy-2* and (f) *lfy-4* inflorescences look identical to *lfy-3*. (g) Average number of cauline leaves/bracts on the main stem of *C. hirsuta* wild type and *lfy* mutant alleles (up to a maximum of 15 leaves were scored). Data reported as means ± SE. (h) *Cardamine hirsuta LFY* gene model showing the *lfy-2*, *lfy-3* and *lfy-4* mutations. Lines represent introns and rectangles represent exons; regions encoding the DNA-binding domain (dark grey) and the conserved N-terminal domain (light grey) are indicated (Hames *et al.*, 2008; Sayou *et al.*, 2016). (i–p) *Arabidopsis thaliana* inflorescences and flowers of the following genotypes: (i, j) wild type; *lfy-6* showing (k) an incomplete floral to shoot transformation with flowers consisting of sepals and (l) a central carpel **[Author, please confirm adjusted placement of '(k)' and '(l)' refer to correct text]**; (m, n) *ap1-1*; (o, p) *lfy-6 ap1-1* showing a complete floral to shoot conversion. Bars: (a, c, e, f, i, k, m, o) 2 mm;(b, d, j, l, n, p) 1 mm.

Fig. 2 LFY function is conserved between *Arabidopsis thaliana* and *Cardamine hirsuta*. (a) *In situ* hybridization on a longitudinal section through a wild-type *C. hirsuta* inflorescence probed with *C. hirsuta LFY*. (b) *Cardamine hirsuta LFY* expression in inflorescence compared with leaf tissue of *C. hirsuta*, determined by quantitative real time polymerase chain reaction (RT-PCR) **[Author, please confirm inserted text 'real time polymerase chain reaction' is correct]** and expressed as fold change (Student's *t*-test: $P = 0.006$). Data reported as means of three biological replicates ± SE. (c, d) *35S::AtLFY* promotes early flowering and converts each axillary shoot to a solitary flower (arrows) in (c) *C. hirsuta lfy-3* and(d) *A. thaliana lfy-6*. (e) *Cardamine hirsuta LFY* and *AP1* expression in 8-d-old *C. hirsuta* seedlings of *35S::AtLFY* compared with 8-d-old wild-type (WT) seedlings, determined by quantitative RT-PCR and expressed as fold change (Student's *t*-test: $P = 0.0008$ for

LFY, $P = 0.029$ for *AP1*). Data reported as means of three biological replicates ± SE. Note that the *lfy-3* allele is segregating in *35S::AtLFY* plants. (f) Average number of cauline leaves/bracts on the main stem of *C. hirsuta* wild type, *lfy-3* and *lfy-3; pAtLFY::AtLFY* genotypes (up to a maximum of 20 leaves were scored). Data reported as means ± SE. (g–i) Whole plant and (j– l) inflorescences of the *C. hirsuta* genotypes: (g, j) wild type, (h, k) *lfy-3* and (i, l) *lfy-3; pAtLFY::AtLFY*. Note that the rosette is omitted from the plant in (h), and older flowers are dissected off the inflorescence in (l). Significance levels: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$. Bars: (a) 50 μm; (c, d, g–i) 1 cm; (j–l) 0.5 cm.

Fig. 3 Regulatory divergence of *AP1*. (a, b) *In situ* hybridizations on longitudinal sections through *Cardamine hirsuta* inflorescences of the following genotypes: (a) wild type and (b) *lfy-3* probed with *C. hirsuta AP1*. In *C. hirsuta* wild type, the expression of *AP1* marks cells at the periphery of the shoot apical meristem that have acquired floral meristem fate and mRNA for this gene accumulates throughout early floral meristems (a). No *AP1* expression is observed in *C. hirsuta lfy* (b). (c) Number of floral organs in wild type and *ap1-119 C. hirsuta* plants showing fewer petals and the presence of ectopic flowers in *ap1-119*. Data reported as means ± SE. (d) *Cardamine hirsuta AP1* gene model showing the positions of *ap1-119* and *ap1-797* mutations. Lines represent introns and rectangles represent exons. (e–h) Scanning electron micrographs (SEM) of (e) wild-type and (f) *ap1-119* inflorescences, and (g, h) *ap1-119* flowers; arrows indicate ectopic flower in the axil of (g) a medial sepal and (h) stipules flanking an aborted lateral sepal **[Author, please confirm amended placement of '(g)' and '(h)' refer to correct text]**. (i–m) Inflorescences of *C. hirsuta* (i) wild type, (j) *ap1-119*, (k) *lfy-2*, (l) *lfy-2;ap1-119* and (m) *lfy-2*;*35S::AtAP1*. (n, o) *Cardamine hirsuta lfy-2*;*35S::AtAP1* flowers consisting of sepals and a central carpel, arrow indicates sepal identity of epidermal cells and arrowhead indicates carpel with stigmatic papillae and ovules (o). (p) *lfy-3* flower consisting of leaves that lack floral organ identity. Bars: (a, b, e–g, o, p) 100 μm; (h) 20 μm; (i–m) 0.5 cm; (n) 0.5 mm.

Fig. 4 *LFY* regulates heteroblastic progression of leaf shape in *Cardamine hirsuta*. (a) Heteroblastic leaf series of *C. hirsuta* wild-type, *lfy-3* and *35S::LFY* genotypes. First to last rosette leaves shown from left to right, rectangles indicate the last rosette leaf; cauline leaves are underlined. (b) Leaflet number is significantly lower in *lfy-3* and

35S::LFY leaves compared with wild type from leaf 3 onwards and the maximum number of leaflets produced in *lfy-3* leaves is significantly lower than wild type. n = 11 (wild-type), 13 (*lfy-3*), 7 (*35S::LFY*). (c) Flowering time does not differ significantly between *lfy-3* and wild type (WT) but *35S::LFY* plants flower early, indicated by the number of rosette leaves produced; significant differences between means are shown by different letters ($P < 0.01$ Tukey's test), n = 10 (WT), 13 (*lfy-3*), 7 (*35S::LFY*). (d) The *y*-axis shows the shape model for the first Eigenshape axis (ES1). ES1 describes the heteroblastic change in terminal leaflet morphology from kidney-shaped (low ES1) to wedge-shaped (high ES1) and accounts for 10.3% of shape variation between all genotypes. The terminal leaflet of *lfy-3* leaves has lower ES1 values that differ significantly from other genotypes from leaf 8 onwards, indicating a delay in heteroblastic development and a failure to acquire final adult shape. The terminal leaflet of *35S::LFY* leaves has higher ES1 values that differ significantly from other genotypes at leaf 1 and from leaf 5 onwards, indicating a precocious acquisition of adult shape. n = 6 (WT and *lfy-3*), 5 (*35S::LFY*). (e) *Arabidopsis thaliana* leaf shape (as measured by the leaf dissection index) of the last rosette leaf before flowering does not differ significantly between Col-0 and *lfy-10* (P = 0.5 Student's *t*-test), n = 5 (Col-0), 14 (*lfy-10*). Bars: (a) 1 cm; (e) 0.5 cm. Statistical tests used in (b–d) were ANOVA with post hoc Tukey tests. Data reported as means ± SE.

Fig. 5 *LFY* is required for *UFO* function in *Cardamine hirsuta*. Plants and representative leaves of (a–d) *35S::UFOi* and (e, f) *35S::UFOi lfy-2* after ethanol induction. Ethanol induction of *UFO* expression, driven by the CaMV *35S* promoter, produces more complex leaves. For example, leaflets dissected to deep lobes (asterisk, b), leaflets initiated in the axils between leaflet and rachis (arrow, c), intercalary leaflets borne on the rachis (arrow, d) and individual leaflets borne on the petiolule (asterisk, d). Leaf shape is unaffected by ethanol induction of *UFO* expression in a *lfy* background (e, f). (g) Number of rosette leaves at flowering time is not significantly affected by ethanol induction of *UFO* expression in *35S::UFOi* lines (Wilcoxon test, $P > 0.05$). Data reported as means ± SE. Bars, 1 cm.