The striking *flower-in-flower* phenotype of *Arabidopsis thaliana* Nossen (No-0) is caused by a novel LEAFY allele

Anne Mohrholz¹, Hequan Sun², Nina Glöckner¹, Sabine Hummel¹, Üner Kolukisaoglu¹, Korbinian Schneeberger², Klaus Harter¹*

¹Department of Plant Physiology, Center for Plant Molecular Biology, Universität Tübingen, 72076 Tübingen, Germany
²Department of Plant Developmental Biology, Max Planck Institute for Plant Breeding Research, 50829 Cologne, Germany

*For correspondence (e-mail Klaus.harter@zmbp.uni-tuebingen.de)*

Summary

The transition to reproduction is a crucial step in the life cycle of any organism. In *Arabidopsis thaliana* the establishment of reproductive growth can be divided into two phases: In the first phase, cauline leaves with axillary meristems are formed and internode elongation begins. In the second phase, lateral meristems develop into flowers with defined organs. Floral shoots are usually determinate and suppress the development of lateral shoots. Here, we describe a *Ds* transposon insertion mutant in the Nossen (No-0) accession with severe defects in floral development and flower morphology. The most striking aspect is the outgrowth of stems from the axillary bracts of the primary flower carrying terminal secondary flowers. Therefore, we named this mutant *flower-in-flower* (*fif*). However, the insertion of the transposon in the annotated gene is not responsible for the *fif* phenotype. By means of classical and genome sequencing-based mapping, the mutation responsible for the *fif*
phenotype was found to be in the LEAFY (LFY) gene. The mutation, a G-to-A exchange in the second exon of LFY, creates a novel Ify allele and causes a cysteine-to-tyrosine exchange in the α1-helix of the LFY DNA-binding domain. Whereas subcellular localization and homomerization are not affected, the DNA-binding of LFYFIF is abolished. We propose that the amino acid exchange interferes with the cooperative binding of LFY to its target DNA. To generate the strong fif phenotype, LFYFIF may act dominant-negatively by either forming non-binding LFY/LFYFIF heteromers or by titrating out the interaction partners, required for LFY function as transcription factor.

Keywords: Arabidopsis thaliana, floral development, flower morphology, Ds transposon, classical/sequencing-based mapping, LEAFY, DNA-binding

Significant Statement: The fif phenotype of Arabidopsis thaliana No-0 is caused by a novel allele of the LEAFY gene

Introduction

The development of flowers is indispensable for the reproductive success of angiosperm plants. During vegetative growth, the shoot apical meristem (SAM) develops leaves and/or branches, the latter with their own SAMs. After the switch to reproductive growth, the apical meristems give rise to flowers. Floral development differs crucially from vegetative shoot growth, as the flower possesses several types of organs of which the number, arrangement and morphology are species-specific. Furthermore, the development of lateral shoots is inhibited in flowers and floral shoots are determinate after the last reproductive organs have been initiated (Piñeiro...
and Coupland, 1998; Ma, 1998; Pidkowich et al., 1999). Thus, the coordination of complex molecular processes is necessary for successful floral development.

There has been significant progress in recent years towards understanding the molecular mechanisms underlying flower formation. Central to this was the identification and cloning of the genes that initiate and maintain floral development in plant species, including Arabidopsis thaliana. The most intriguing discovery was the Arabidopsis loss-of-function mutants with structures that are intermediate between floral and vegetative shoots. The cloning of the corresponding genes revealed the existence of the master regulators required for the floral initiation process (FLIP). To date, five FLIP regulatory master genes are known: LEAFY (LFY), APETALA1 (AP1), CAULIFLOWER (CAL), APETALA2 (AP2) and UNUSUAL FLORAL ORGANS (UFO) (Pidkowich et al., 1999). LFY and AP1 play a primary role in initiating the floral program, as the corresponding loss-of-function mutants do not generate shoots with floral characteristics and the ectopic expression of either gene induces precocious flower formation (Irish and Sussex, 1990; Huala and Sussex, 1992; Bowman et al., 1993). Based on its amino acid similarity and expression characteristics CAL appears to be functionally redundant to AP1 (Kempin et al., 1995). LFY, AP1 and CAL encode for transcription factors and are expressed predominantly in floral primordia (Weigel et al., 1992; Mandel et al., 1992; Kempin et al., 1995).

During plant vegetative growth, LFY expression increases in newly formed leaves until a certain threshold is reached (Bowmann et al., 1993). LFY then induces the expression of AP1/CAL genes by binding to the AP1/CAL promoters. Through their mutual transcriptional up-regulation, LFY and AP1/CAL cooperate to cause the floral transition (Blazquez et al., 2006). Once the floral meristem is established, the FLIP gene functions govern its spatial patterning by inducing the expression of the floral homeotic ABC genes, such as AP2, AP3, Pistillata (PI) and AGAMOUS (AG). The
ABC gene functions in turn control the identity of the stereotypically arranged Arabidopsis floral organs (Coen and Meyerowitz, 1991; Lohmann and Weigel, 2002). In the course of our study of the influence of abiotic stress on flower symmetry, we searched for novel insertion mutants with defects in floral development or morphology in different Arabidopsis thaliana accessions. We focused on genes that had not yet been linked to flowering. A Ds transposon insertion mutant, which developed secondary inflorescences with partially aberrant flowers, was identified in the No-0 accession. The wild-type allele of the gene carrying the Ds transposon codes for a cystein/histidine-rich C1 domain protein (Shinya et al., 2007; Miwa et al., 2008). However, a thorough genetic analysis revealed that the transposon-inserted allele is not the cause of the observed floral phenotype. Using classical mapping and mapping-by-sequencing, we eventually found a novel mutant allele of LFY to be responsible for the aberrant floral development and flower morphology and determined the molecular reason for LFY malfunction.

**Results**

The flower-in-flower (fif) transposon insertion line displays a novel flower phenotype

In order to identify novel Arabidopsis thaliana mutants with defects in flowering we screened the RIKEN Arabidopsis Phenome Information Database (RAPID; Kuromori et al., 2006). RAPID also covers a Ds transposon mutant collection in the Arabidopsis Nossen-0 (No-0) background (Ito et al., 2002; Kuromori et al., 2004). We identified a transposon-tagged line (15-3794-1), which developed secondary inflorescences with partially aberrant flowers (Fig. 1a). Because of this phenotype, we named this novel Arabidopsis mutant flower-in-flower (fif).
As shown in Figure 1c and d, wild-type *Arabidopsis* flowers does not have bracts but consist of four concentric rings of 4 sepals, 4 petals, 6 stamens and 2 fused carpels. In contrast, the primary flower of the *fif* mutant had bracts as well as sepals but the petals were incompletely developed or entirely missing (Fig. 1b, e). In addition, there were either no stamens or the stamens displaying an aberrant development (Fig. 1b, e). Furthermore, there were more than 2 carpels per flower, which were not or only partially overgrown and did not establish fertile ovaries. Most obvious, however, was the outgrowth of stems from the axillary meristems of the bracts, which carried terminal secondary flowers. A few secondary *fif* flowers showed a wild-type-like phenotype and were, thus, fertile (Fig. 1b, e).

Furthermore, the *fif* mutant plant displayed a bushy habitus compared to wild-type No-0 (Fig. 2a, b). This bushy appearance was due to an enhanced number of stem-born side branches compared to wild-type No-0, whereas the number of rosette-born side shoots was the same in *fif* and wild-type No-0 plants (Fig. 2c). In addition, *fif* mutant plant exhibited delayed flowering compared to wild-type No-0 (Fig. 2a, b).

**The transposon insertion is not responsible for the *fif* phenotype**

According to the RIKEN RAPID and our own genotyping results, the *Ds* transposon was located in the second exon of the gene *At1g20990* that codes for a putative cysteine/histidine-rich C1 domain protein with an as yet unknown function. To validate the causal relationship between the *fif* phenotype and the *Ds* transposon insertion, we analysed an independent insertion mutant in the *Arabidopsis thaliana* Col-0 background, which exhibited a T-DNA insertion in the promoter region of *At1g20990* (SALK_073291; Alonso *et al.*, 2003). However, homozygous mutant plants of this line showed no aberrant phenotype compared to wild-type (Col-0) with respect to floral development, flower morphology, flowering time and growth habitus.
This observation raised doubts as to whether there is a functional link between the Ds transposon insertion and the fif mutant phenotype. We therefore performed a (co-) segregation analysis by backcrossing the fif mutant with wild-type No-0 in both directions (♀ fif x ♂ No-0, ♀ No-0 x ♂ fif). Irrespective of the direction, the crosses were successful as demonstrated by PCR on genomic DNA extracted from F1 plants using Ds transposon- and At1g20990-specific primers (Figure S1). All tested F1 plants were heterozygous for the Ds transposon and wild-type At1g20990 and displayed wild-type floral organs and growth habit (Figure S1). Therefore, the mutation that causes the fif phenotype is recessive. Next, six F1 plants were self-fertilized and 20 to 30 progenies each analysed for their pheno- and genotypes. As shown in figure 3, around one quarter of the F2 plants displayed the fif phenotype indicating that it is caused by a single mutant gene. Intriguingly, our genotyping results showed that the Ds transposon insertion did not co-segregate with the fif phenotype: 29 % of the fif phenotype-displaying plants did not contain the transposon, an additional 49% contained the transposon insertion only heterozygously (Figure 3). These results prove that the Ds insertion into the At1g20990 locus does not cause the fif phenotype.

The fif phenotype is caused by a novel allele of LEAFY (LFY)

To identify the mutant locus genetically responsible for the fif phenotype, we combined a classical mapping (Neff et al., 2002; Kover et al., 2009; Pacurar et al., 2012) with a mapping-by-sequencing approach (James et al., 2013; Schneeberger, 2014). To establish a mapping population, fif mutant plants (No-0) were crossed in both direction with plants of the Col-0 accession. Irrespective of the crossing direction, all the F1 plant displayed a wild-type phenotype (Figure S2a). Eight F1 plants were self-fertilized and 1582 F2 plants characterized phenotypically.
accordance with the self-crossing results described above, around 25 % of the F2 plants (437 of the 1582) showed the fif phenotype (Figure S2b). Leaf material was harvested from 425 of the 437 F2 plants in groups of 15 to 20 individuals; in addition leaf material from 200 F2 plants was collected individually. Genomic DNA was extracted and used for classical mapping. Using chromosome-specific INsertion and DELetion (INDEL) markers (Pacurar et al., 2012) the mutant locus was mapped to the q-arm of chromosome 5 (Figure 4a). Two additional INDEL markers and two Single Nucleotide Polymorphism (SNP) based Derived Cleaved Amplified Polymorphic Sequences (dCAP) markers (Kover et al., 2009; Neff et al., 2002) limited the Quantitative Trait Locus (QTL) responsible for the fif phenotype to the terminal end of chromosome 5’s q-arm (Figure 4b, dCAP S5-24: 99% No-0).

To establish the exact localization of the mutant locus, we deep-sequenced the total genome of 245 homozygous fif mutant plants derived from the fif (No-0) x WT (Col-0) crosses described above, and determined the frequencies of No-0 and Col-0 alleles along the chromosomes. Whereas the heterozygous distribution of No-0 and Col-0 sequences was found to be equal with respect to chromosomes 1 to 4 (Figure S3a-d), there was a very significant deviation towards No-0 sequences at the terminal end of chromosome 5 (Figure 5a). A detailed examination of this 300 kb stretch revealed 100 % identity with the No-0 sequence (Figure 5b). This sequence stretch conformed with the QTL identified by classical mapping.

A detailed comparison of the fif and wild-type No-0 sequence in this 300 kb stretch revealed a single SNP, which did not result in a silent mutation but caused a change in a codon. This SNP was also found in all the 143 individually tested fif mutant plants and reflected a single guanine-to-adenine exchange in the second exon of the LEAFY (LFY) gene (At5g61850, Figure 5c). This mutation caused a cysteine-to-tyrosine amino acid exchange at position 263 in the DNA-binding domain of the LFY
protein (Figure 5d). To prove that this point mutation causes the fif phenotype, we transformed the fif mutant (No-0) with constructs expressing LFY-GFP or LFY\textsuperscript{FIF}-GFP under the control of the 35S promoter. Whereas the expression of LFY-GFP complemented the fif mutant phenotype almost completely, there was no complementation with LFY\textsuperscript{FIF}-GFP (Figure S4).

**LFY\textsuperscript{FIF} impairs DNA-binding capability but shows wild-type intracellular localization and homomerization**

Having identified a new LFY allele to be responsible for the fif phenotype, we next analysed the putative consequences of the Cys263-to-Tyr exchange for LFY protein properties at molecular and cell biological levels.

To test a putative alteration in subcellular localization, C-terminal GFP fusions of wild-type LFY and the mutant LFY version (LFY\textsuperscript{FIF}) were expressed under the control of the *Arabidopsis ubiquitin 10* (*UBQ10*) promoter in transiently transformed *Nicotiana benthamiana* epidermal leaf cells. The functionality of C- (and N-terminal) GFP fusions of LFY was previously shown by the genetic complementation of the *lfy-12* mutant phenotype (Wu et al., 2003). As shown in figure 6a, LFY-GFP and LFY\textsuperscript{FIF}-GFP localised to the cytoplasm and the nucleus in a similar manner. The observed fluorescence pattern of LFY-GFP and LFY\textsuperscript{FIF}-GFP is in accordance with the pattern previously reported for their expression in tobacco epidermal leaf cells (Siriwardana and Lamb, 2012b).

Next, we tested by *in vivo* FRET-FLIM whether LFY protein-protein interaction, here especially LFY homomerization (Sirwardana and Lamb, 2012a), was altered. To do so, C-terminal GFP fusions (FRET donor) and C-terminal RFP fusions (FRET acceptor) were transiently expressed, either individually (donor only) or in combination in *N. benthamiana* epidermal leaf cells and the fluorescence lifetime of
the donor fusion was measured. As shown in figure 6b, the fluorescence lifetimes of LFY-GFP and LFYFIF-GFP were similar in the absence of the acceptor fusions. However, the lifetimes of LFY-GFP and LFYFIF-GFP decreased significantly when they were co-expressed with either LFY-RFP or LFYFIF-RFP demonstrating homotypic (LFY-LFY, LFYFIF-LFYFIF) and heterotypic (LFY-LFYFIF) homomerization in planta (Figure 6b). In addition, there was no significant difference in the interaction of the homotypic and heterotypic homomers (Figure 6b).

The Cys263-to-Tyr exchange is located in the first α-helix of the LFY DNA-binding domain (Figure 5d). We, therefore, used a quantitative DNA-protein interaction ELISA approach (qDPI-ELISA; Fischer, Böser et al., 2016) to test whether the mutation interferes with the DNA-binding capability of LFY in vitro. We expressed N-terminally GFP-tagged full-length LFY, as well as full-length LFYFIF and GFP, in E. coli independently and applied the crude extracts containing the fusion proteins or GFP, in identical amounts, based on the GFP fluorescence and western-blotting, to ELISA plates in two dilutions. The plates were covered with double-stranded (ds) DNA oligonucleotides representing either the LFY-binding sequence of the AP1 promoter (pAP1), a mutated pAP1 version (pAP1m) that is not recognized by LFY (Winter et al., 2011) a random sequence without any similarity to the LFY binding motif (C28M12), or were uncovered. The DNA-binding efficiency of the proteins was recorded by determining the GFP fluorescence of the bound proteins (Fischer, Böser et al., 2016). GFP-LFY exhibited a specific binding to pAP1 and no binding to any other oligonucleotide or to the oligonucleotide-free ELISA plate (Figure 7). In contrast, GFP-LFYFIF, like GFP or the E. coli crude extract without recombinant protein, was unable to recognize pAP1 or any other oligonucleotide (Figure 7). To exclude the possibility that the Cys263-to-Tyr exchange may alter the DNA-binding specificity we used a DPI-ELISA based approach to screen a dsDNA oligonucleotide
library reflecting 4096 randomized DNA hexamers (Brand et al., 2013a, b) with GFP-LFY- and GFP-LFY\textsuperscript{FIF}-containing \textit{E.coli} extracts. Whereas a DNA-binding consensus sequence was obtained for GFP-LFY (5′-GGGC-3′/3′-CCCG-5′), there was no DNA-binding of GFP-LFY\textsuperscript{FIF} to any oligonucleotide in the library.

Discussion

In our search for novel floral genes in \textit{Arabidopsis thaliana} we identified the \textit{fif} \textit{Ds} transposon insertion mutant in the No-0 accession in the RIKEN RAPID collection (Ito et al., 2002; Kuromori et al., 2004). \textit{fif} mutant plants display a novel floral phenotype and inflorescence architecture, as they develop aberrant and infertile primary flowers in combination with short stems that emerge from vegetative meristems in the axillars of the bracts and carry fertile secondary flowers.

The \textit{Ds} transposon insertion in the genome of the \textit{fif} mutant was annotated to gene \textit{At1g20990}, which encodes a cysteine/histidine-rich C1 domain protein. However, as demonstrated by our genetic analysis, the \textit{Ds} transposon insertion into the \textit{At1g20990} locus is not the cause of the \textit{fif} phenotype. Obviously, another mutant locus generated somewhere else in the genome, most likely during transposon movement, is responsible for the \textit{fif} phenotype. Using combined classical and genome sequencing-based mapping approaches, the causal mutation for the \textit{fif} phenotype was found to be in the \textit{LFY} gene. The mutation is a single G-to-A exchange in the second exon of \textit{LFY}, creating the novel, recessive \textit{lfy} allele. The mutation causes a Cys-to-Tyr exchange at position 263 in the LFY\textsuperscript{FIF} amino acid sequence.

The cell biological analysis of LFY-GFP and LFY\textsuperscript{FIF}-GFP revealed an intracellular localization in the cytoplasm and nucleus of tobacco epidermal leaf cells identical to that previously reported for LFY-GFP (Siriwardana and Lamb, 2012a). Thus, a mis-
localisation cannot be the cause of the LFY\textsuperscript{FIF} malfunction. In addition, as shown by quantitative FRET-FLIM interaction studies the mutation does not interfere with the homomerization capacity of LFY. Especially the latter result was to be expected as the domain essential for homomerization is located at the N-terminus of LFY (amino acid 46 to 127; Siriwardana and Lamb, 2012a).

However, our quantitative DPI-ELISA assay demonstrated that, in contrast to LFY-GFP, LFY\textsuperscript{FIF}-GFP lost its capacity to bind to its DNA target, as it is present, for instance, in the \textit{AP1} promoter (Winter et al., 2011). Furthermore, the DPI-ELISA based approach for the determination of putative alterations in binding specificity did not reveal any DNA-binding activity for LFY\textsuperscript{FIF}-GFP.

According to the available crystal structure of the DNA-bound dimer, Cys263 is well conserved between the LFY homologs of many plant species but has never previously been reported to be crucial for DNA-binding (Hames et al., 2008). Intriguingly, Cys263 does not contribute to the physical contact of LFY with DNA; however, the \(\alpha1\)-helix, in which Cys263 is positioned, participates in the cooperative DNA-binding of LFY, as it facilitates the establishment and stabilization of the DNA-binding domains in the minor and major groove of DNA (Hames et al., 2008).

Therefore, the change of the relatively small Cys to the bulky, aromatic Tyr might prevent the folding of the \(\alpha1\)-helix and thereby strongly restrict the cooperative binding of LFY to its target DNA.

The total failure of LFY\textsuperscript{FIF} to bind to DNA explains the strong floral phenotype of especially the primary flowers. LFY is one of the master regulators in the FLIP of \textit{Arabidopsis} (and other plant species) and controls, together with other factors and \textit{via} a complex regulatory network, the spatiotemporal expression of downstream FLIP genes and also of the homeotic flower genes required for flower organ formation. Although only a single amino acid exchange is affected, LFY\textsuperscript{FIF} mirrors in principle
the flower phenotype of known strong lfy alleles. However, of the more than 15 described lfy alleles (Weigel et al., 1992), the six alleles that show such a strong floral phenotype produce shortened LFY polypeptides caused by either premature stop codons (lfy-1, lfy-6, lfy-7, lfy-8, lfy-11) or a non-sense frame shift C-terminal of Gln196 (lfy-15). Hence, the strong phenotype of the fif allele needs a different explanation: LFYFIF may act dominant-negatively by either forming non-functional heteromers with wild-type LFY, which cannot longer bind to DNA, or by titrating out interaction partners required for LFY function (Siriwardana and Lamb, 2012b). However, as long as sufficient wild-type LFY is present in heterozygous plants, the fif mutant shows recessive inheritance.

The failure of LFYFIF to bind to DNA is also explains the bushy growth architecture of the fif mutant. It has recently been shown (Chahtane et al., 2013) that mutations in lfy can cause the emergence of axillary meristems instead of floral meristems resulting in an enhanced number of side branches. In addition, the ectopic expression of a nearly full-length LFY version with weaker in vitro DNA-binding capacity and dramatically reduced in vivo transcriptional activity [LFYHARA(Δ40)] in the Col-0 accession causes a bushy phenotype similar to that of the No-0 fif mutant (Chahtane et al., 2013). Interestingly, the His387-to-Ala and Arg390-to-Ala in LFYHARA(Δ40) are also mooted to interfere with the cooperative binding of LFY to its target DNA as well.

Taken together, our data demonstrate the general importance of Cys263 for LFY function not only in floral development but also in axillary meristem outgrowth in Arabidopsis.

Most intriguingly, the fif floral phenotype appears to be specific for the No-0 accession, as, to our knowledge, it has never been reported for the Col-0 or any other accession. However, the fif phenotype also becomes also manifest in the Col-0 accession when the fif locus of No-0 is transferred to Col-0. This phenomenon might
be explained by differences in the spatio-temporal transcriptional activity of the No-0 and Col-0 LFY loci during vegetative meristem and floral development. Therefore, the fif phenotype may only be visible in other accessions such as Col-0 when the No-0 locus is artificially introduced into them and drives LFY\textsuperscript{FIF} accumulation.

**Experimental procedures**

**Plant material**

Seeds of the homozygous Ds transposon insertion line 15-3794-1 and the corresponding wild-type accession (No-0) were obtained from the RIKEN Arabidopsis Phenome Information database (RAPID; Kuromori et al., 2006). Seeds of the homozygous T-DNA insertion line Salk_073291 and the corresponding wild-type accession (Col-0) were obtained from the Nottingham Arabidopsis Stock Centre (NASC; Alonso et al., 2003).

**Plasmid construction**

Using gene-specific primers [sense (S): 5´-caccATGGATCCTGAAGGTTTCACG-3´, antisense (A): 5´-GAAACGCAAGTCGTCGCCG-3´] the cDNA of LFY was amplified from pSST14 (gift Jan Lohmann, University of Heidelberg, Germany) and cloned in pENTR™/D-TOPO®. Site-directed mutagenesis (SDM) was performed to produce the fif cDNA using the following primers (S: 5´-CTGTTCACCTGTACGAACAATaCCGTGAGTTCCTTCTTCAG-3´, A: 5´-CTGAAGAAGGAACTCACGGtATTGTTCGTACAAGTGGAACAG-3´). With Gateway™ LR Clonase™ II Enzyme mix the LFY cDNA was inserted into pUGT1-Dest (A. Hahn, unpublished) and pB7RWG2-Dest (Karimi et al., 2002) for plant expression and into pET-Dest42GFP (Fischer, Böser et al., 2016) for E. coli expression.
Classical mapping and mapping by genome sequencing

Genetic mapping was accomplished using 100 phenotypic fif plants collected from a F2 population derived from a cross between fif (No-0) and Col-0. The mapping strategy and the molecular markers used to identify the causal locus were described by Păcurar et al. (2012). After mapping of the chromosome arm and next-generation sequencing (NGS, see below) the point mutation was confirmed by derived cleaved-amplified polymorphic sequence primers designed by using the dCAPS Finder 2.0 software (Neff, Turk and Kalishman, 2002). One or two mismatches were introduced in one of the used primer to incorporate an allele-specific restriction site into the PCR product. After amplification, the PCR products were digested (enzymes from Thermo Scientific) following the manufacturer's recommendations and separated on a 4% agarose gel. All used markers are listed in table S1.

NGS mapping was performed using a pool of 425 phenotypic fif plants from the crossing described above. A pool of 40 wild-type No-0 plants was sequenced to generate a genome-wide marker list and to mine the fif genome for acquired mutations. Isolation of genomic DNA was performed in groups up to 20 plants using the DNeasy® Plant Mini Kit (QIAGEN) following the manufacturer's recommendations. DNA concentration was determined with the use of NanoDrop ND-1000 and the whole pool composed by using 100 µg DNA of each group.

Sequencing was performed at the Max Planck-Genome-Centre Cologne by a HiSeq2500 (Illumina) Sequencer producing ~35.000.000 read-pairs for each pool. Short reads of both pools were respectively aligned against the Col-0 reference sequence (TAIR10) and SNPs were called using shore pipeline (version v0.8) with GenomeMapper (version v0.4.4s) with default parameters (Ossowski et al., 2008; Schneeberger et al., 2009a). Genome-wide SNP markers were defined with filtering for sequencing coverage and allele frequency using SHOREmap (version 3.0, Sun et
Sliding window-based estimation of allele frequencies of the Nos allele in the pooled F2 samples and identification of a mapping interval were performed with SHOREmap (version 3.0) using default parameters. Comparison of the consensus calls of both pools in the 300 kb mapping interval revealed the mutation in LFY.

Localization and FRET-FLIM studies

The indicated constructs and p19 as gene silencing suppressor were transformed into *Agrobacterium tumefaciens* strain GV3101 and infiltrated into *Nicotiana benthamiana* leaves. The localization of the fusion proteins was performed 3 days after infiltration using 488 nm or 561 nm lasers for GFP or RFP excitation, respectively, at the SP8 laser scanning microscope (Leica Microsystems GMBH) with LAS AF and SymPhoTime software using a 63x/1.20 water immersion objective (Ladwig *et al.*, 2015). FLIM data were derived from measurements of at least 20 probes for each fusion protein combination. To excite LFY-GFP and LFYFIF-GFP for FLIM experiments, a 470 nm pulsed laser (LDH-P-C-470) was used, and the corresponding emission was detected with a SMD Emission SPFLIM PMT from 495 to 545 nm by time-correlated single-photon counting using a Picoharp 300 module (PicoQuant). Each time-correlated single-photon counting histogram was reconvoluted with the corresponding instrument response function and fitted against a monoexponential decay function for donor-only samples and a biexponential decay function for the other samples to unravel the GFP fluorescence lifetime of each probe. The average GFP fluorescence lifetimes as well as the standard error values were calculated using Microsoft Excel 2013. To test for homogeneity of variance Levene’s test (df=5/140, F=26.298, p < 0.0001) was used and statistical significance
was calculated by a two-tailed, all-pair Kruskal-Wallis test followed by a Steel-Dwass post hoc correction using JMP version 12.2.0 (Ohmi et al., 2016).

**qDPI-ELISA, DPI-ELISA based screening and western blotting**

qDPI-ELISA was performed using *E. coli* crude extracts containing GFP-tagged LFY or LFYFIF, GFP alone or no fluorescent protein according to Fischer, Böser et al. (2016). The sequences of the 5’-biotinylated dsDNA oligonucleotides *AP1, mAP1* and *C28M12* used for the immobilization on Streptavidin-coated 384 well microtiter plate are displayed in table S2. Before addition to the microtiter plate, the equal content of GFP-tagged fusion protein in the crude extracts was adjusted according to the GFP fluorescence using a fluorescence reader (TECAN Safire).

The DPI-ELISA based specificity screening, using a dsDNA oligo array on a 384 well microtiter plate covering all possible 4096 hexanucleotide DNA motifs was performed as described previously (Brand et al. 2013a, b).

**Acknowledgements**

The authors would like to thank J. Lohmann (Universität Heidelberg, Germany) for the *LFY* cDNA, M. Fischer for technical support, J. Schröter and G. Huber for support in plant cultivation, F. de Courcy for proofreading the manuscript and the members of the multidisciplinary graduate school “Morphological Variability of Organisms under Environmental Stress” for discussion. This work was supported by a grant of the Ministerium für Wissenschaft, Forschung und Kunst Baden-Württemberg to A. Mohrholz.

**Supporting information**

Additional Supporting Information is found in the on-line version of this article.
References


Figure legends

Figure 1. Flower phenotype of the Arabidopsis thaliana (No-0) flower-in-flower (fif) mutant. (a) Overview over representative fif mutant “inflorescence” displaying different flower types 1 to 4. (b) Floral organs of the primary fif flower (1) and different secondary fif flowers (2-4). (c) Flower of the wild-type No-0 accession. (d-e) Flower diagram of the wild-type No-0 flower (d) and the primary flower of the fif mutant (e). (f) Primary flowers of the fif mutant with stems that outgrow from axillary bract meristems (red arrow heads) and carry secondary flowers. Size bar: 1 mm.

Figure 2. Growth habitus and degree of branching of wild-type No-0 and fif mutant plants. (a-b) Overview over the growth habitus and magnification of the inflorescence of 6.5-weeks old wild-type No-0 (a) and fif (b) plants, grown side-by-side in the greenhouse. Size bar: 1.0 cm. (c) Number of rosette-born side branches and stem-born side branches of wild-type No-0 (white bars) and fif (black bars) plants. Error bars indicate the standard deviation of the mean (n_{No-0} = 33, n_{fif} = 25, ***: p = 2x10^{-23}).

Figure 3. Segregation of the floral phenotype and the Ds transposon insertion within the combined F2 population of (♀ fif x ♂ No-0) and (♀ No-0 x ♂ fif) backcrosses. (a) Distribution of F2 plants, showing either the wild-type (78.4 %) or the fif floral phenotype (21.6 %). (b) Distribution of the transposon insertions within the plants of the F2 population that displayed the fif floral phenotype. White circle cutout: no transposon insertion (29.2 %), striped cutout: heterozygous for the Ds transposon insertion (45.8 %), black cutout: homozygous for the Ds transposon insertion (25.0 %).
Figure 4. INDEL marker- and SNP-based dCAP marker-associated containment of the *fif* locus using a mapping population generated by a cross of the *fif* mutant (No-0) with wild-type Col-0. (a) Schematic representation of the 5 *A. thaliana* chromosomes (sizes in MB) and the localization of the chromosome-specific INDEL markers initially used for mapping (codes above blue lines). (b) Schematic representation of the q-arm of chromosome 5 and the localization of INDEL (codes above the blue lines) and SNP-based dCAP markers (codes above red lines) used for fine mapping. The pie charts show the distribution of the No-0 and Col-0 genotypes for each chromosome (a) and the q-arm of chromosome 5 (b). White circular outcut: homozygous for Col-0, striped outcut: heterozygous for Col/No-0, black outcut: homozygous for No-0; red dot: localization of the centromere.

Figure 5. Identification of the *fif*-related SNP in the second exon of the *LEAFY (LFY)* locus on chromosome 5 by genome sequencing of a mapping population generated by a cross of the *fif* mutant (No-0) with wild-type Col-0. (a) Allele frequency analysis of the Nos genotype within chromosome 5 of the recombinant mutant pool. Each red circle refers to a SNP marker distinguishing the Nos and Col genotypes. The blue line refers to a 200 kb sliding window analysis of the allele frequencies. The brown line and blue box highlight the estimated mapping intervals (x-axis: genomic location; y-axis: Nos allele frequency). (b) Like (a), but only showing the 300 kb mapping interval. (c) Exon-intron organization of the *LFY* locus with the *fif*-related SNP marked by an arrow. Exons are shown as grey boxes and introns as exons connecting lines. (d) Sequence of the *LFY* gene showing the *fif* SNP (G to A exchange, red) and the resulting amino acid exchange (C to Y, red) within the DNA-binding domain of the LFY protein. Green boxes: β-sheets; blue boxes: α-helices (according to Hames et al., 2008).
Figure 6. Comparative analysis of the intracellular localization and homomerization capacity of LFY and LFY\textsuperscript{FIF}. (a) Confocal fluorescence images of transiently transformed \textit{Nicotiana benthamina} epidermal leaf cells expressing LFY-GFP and LFY\textsuperscript{FIF}-RFP in the same cell. Size bar: 5 µm. (b) FRET-FLIM analysis of the homo- and heterotypic interaction of LFY and LFY\textsuperscript{FIF}. LFY-GFP or LFY\textsuperscript{FIF}-GFP were expressed either alone or together with the indicated RFP fusions and the fluorescence lifetime of the GFP fusions measured in nucleus. A reduction of the GFP fluorescence lifetime indicates interaction. The data are presented in Box-and-Whisker plots including the median (thick line), the upper and lower quartile (+/- 25%, white boxes), the maximum and minimum (dotted line) and outlier points (n > 20, each). The variance was analyzed by a Levene test and statistical significance was determined with an all-pair, two-sided Kruskal-Wallis test followed by an all-pair Steel-Dwass test (**: p < 0.01; ***: p < 0.001).

Figure 7. Comparative analysis of the \textit{in vitro} DNA-binding capacity of LFY and LFY\textsuperscript{FIF} using a GFP-fluorescence-based DPI-ELISA approach. GFP-LFY and GFP-LFY\textsuperscript{FIF} were expressed in \textit{E.coli}. After extraction, crude extracts containing either no recombinant protein (w/o protein) or, based on GFP fluorescence, equal amounts of GFP or GFP fusion protein were added to ELISA plates covered with either the double-stranded (ds) DNA oligonucleotide \textit{pAP1}, which contains a LFY recognition site, an altered version of \textit{pAP1} (\textit{pAP1m}), in which the recognition site was mutated, a dsDNA oligonucleotide unrelated to the \textit{pAP1} and \textit{pAP1m} sequences (\textit{C28M12}) or without any DNA-oligonucleotide. The amount of DNA-bound fusion protein was detected by reading out the GFP fluorescence. The crude extract was either used undiluted (black bars) or in a 1:4 dilution (grey bars). Error bars indicate the standard deviation of the mean (n = 3) and asterisks statistically significant differences to the
background fluorescence (dotted horizontal line), determined by two-sided t-test (*: p < 0.05; **: p < 0.01).

The inlet shows a Western-blot of the crude extracts using a GFP polyclonal antiserum for detection of GFP, GFP-LFY and GFP-LFY\textsuperscript{FIF} as well as a Coomassie stain as loading control.
The number of rosette-born and stem-born side branches per plant was measured. The graph shows a significant difference between the No-0 and fif genotypes, with fif producing significantly more stem-born side branches compared to No-0.

*** indicates a statistically significant difference.
Nichtparametrische Vergleiche für alle Paare mittels Dunn-Methode für gemeinsame Ränge

* signifikant <0,05
** hoch signifikant <0,01
*** Höchst signifikant <0,001
**Figure Caption**

A bar graph showing the effects of different oligonucleotides on GFP-LFY and GFP-LFYFIF fluorescence. The x-axis represents the oligonucleotide and protein combinations, while the y-axis represents GFP fluorescence (RFU). The graph includes the following treatments:

- **pAP1m C28M12**
- **pAP1**
- **C28M12**
- **GFP-LFYFIF**
- **GFP**

Each treatment is represented by a different color and style of bar, with error bars indicating standard deviation. The graph also includes a loading control. Significant differences are indicated by asterisks (**p < 0.01** and *p < 0.05*).

**Legend**

- **1**
- **1:4**
- **w/o protein**