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Recommended Citation
Risseeuw, Eddy; Venglat, Prakash; Xiang, Daoquan; Komendant, Kristina; Daskalchuk, Tim; Babic, Vivijan; Crosby, William L.; and Datla, Raju, "An Activated Form of UFO Alters Leaf Development and Produces Ectopic Floral and Inflorescence Meristems" (2013). PLoS ONE, 8, 12. https://scholar.uwindsor.ca/biologypub/968

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**An Activated Form of UFO Alters Leaf Development and Produces Ectopic Floral and Inflorescence Meristems**

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**Abstract**

Plants are unique in their ability to continuously produce new meristems and organ primordia. In Arabidopsis, the transcription factor LEAFY (LFY) functions as a master regulator of a gene network that is important for floral meristem and organ specification. UNUSUAL FLORAL ORGANS (UFO) is a co-activator of LEAFY and is required for proper activation of APETALA3 in the floral meristem during the specification of stamens and petals. The ufo mutants display defects in other parts of the flower and the inflorescence, suggestive of additional roles. Here we show that the normal determinacy of the developing Arabidopsis leaves is affected by the expression of a gain-of-function UFO fusion protein with the VP16 transcriptional activator domain. In these lines, the rosette and cauline leaf primordia exhibit reiterated serration, and upon flowering produce ectopic meristems that develop into flowers, bract leaves and inflorescences. These striking phenotypes reveal that developing leaves maintain the competency to initiate flower and inflorescence programs. Furthermore, the gain-of-function phenotypes are dependent on LFY and the SEPALLATA (SEP) MADS-box transcription factors, indicative of their functional interactions with UFO. The findings of this study also suggest that UFO promotes the establishment of the lateral meristems and primordia in the peripheral zone of the apical and floral meristems by enhancing the activity of LFY. These novel phenotypes along with the mutant phenotypes of UFO orthologs in other plant species suggest a broader function for UFO in plants.

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**Introduction**

The continuous production of new meristems is a characteristic feature in plants and accounts for their distinctive indeterminate growth. After germination, the shoot apical meristem (SAM) produces phytomers repetitively which represent an internodal stem with a node comprising of a leaf subtending an axillary meristem [1]. The apical and axillary meristems are usually indeterminate [2], whereas the leaf primordia are most often determinate [3]. During the course of its life cycle, the plant produces multiple meristems and primordia and these will acquire different identities to give rise to different organs. The meristems can switch identity over time and this is particularly important during the transition from the vegetative to the reproductive phase. The identities are determined by the combinatorial expression and functions of specific meristem and organ identity genes controlled spatially and temporally by preprogrammed genetic networks [4]. In Arabidopsis, the LEAFY (LFY) protein is a master regulator of the organ identity genes and its function is essential for both conferring floral meristem identity and the subsequent identity of the individual floral organs. LFY is a plant specific transcription factor and activates several key floral organ identity genes including the ABC class MADS-box genes [5]. The lfy mutant is impaired in the floral fate specification of the meristems produced by the inflorescence meristem, and as a consequence, new meristems default towards a co-inflorescence fate, resulting in a leafy appearance [6,7].

UNUSUAL FLORAL ORGANS (UFO) is a key cofactor of LFY to specify the petal and stamen whorls by regulating the expression of the B-class MADS-box gene APETALA 3 (AP3) in the floral meristem [8,9,10]. Therefore in ufo flowers, petal and stamen development is severely affected resulting in either reduction or complete absence of these organs [11]. The ufo mutant is also associated with a range of additional defects outside the AP3 expression domain including the loss of carpels in some flowers; replacement of flowers by filaments; and perturbed transition of the apical meristem from the vegetative to the inflorescence identity, particularly under short day conditions [12]. These findings suggest that the spatial and temporal overlap of the LFY and UFO expression domains are important for specification of the floral meristem and the floral organ primordia [13]. Compared to Arabidopsis, the expression domains of the LFY and UFO orthologs vary considerably in other plant species [14]. Accordingly their individual functions and their respective mutant phenotypes are quite different from the Arabidopsis lfy and ufo mutants. For example, the UFO orthologs double top (dot) mutant in petunia is unable to produce flowers, whereas in the pea unifoliate (uni) and staminula pistillida (sfp) mutants, the orthologs of LFY and UFO, show reduced leaf complexity in addition to inflorescence abnormalities [15,16]. Though ufo mutants do not affect the leaf
shape in Arabidopsis, the ectopic expression of UFO results in serrated leaves [8]. These phenotypes indicate that the function of UFO and its homologs in other species are not restricted only to the specification of the petal and stamen whorls, but also extended to include other meristems and primordia both inside and outside of the floral program.

UFO belongs to a large group of F-box proteins encoded by a family of over 700 genes in Arabidopsis [17,18]. F-box proteins confer specificity to the SCF (Skp1-cullin-F-box complex) class of ubiquitin ligases by binding and presenting specific target proteins to the ubiquitin conjugating enzyme [19]. The UFO F-box domain is required for the interaction with the Skp1 adapter protein (ASK proteins in Arabidopsis) and the COP9 signalosome [17,20]. The C-terminal domain of UFO has been shown to bind LFY and this interaction likely leads to its ubiquitination [10,15].

Transcription factors are often substrates for ubiquitination where this modification plays a dual role by activation followed by their turnover [21,22,23,24]. Therefore, UFO plays a unique role in flower development by ubiquitinating the plant specific transcription factor, LFY, which likely also requires the participation of UFO in the transcriptional complex [10]. In this study, the involvement of UFO in a transcriptional complex was tested by the construction of activator and repressor versions of UFO employing the well characterized and widely used heterologous VP16 based activator and Engrailed based repressor domains respectively. The developmental effects produced by these overexpression constructs were evaluated in transgenic Arabidopsis, Brassica napus and tobacco plants. Our results showed that especially the activator fusion had a dramatic effect on the UFO gain-of-function phenotypes that include development of ectopic flowers and inflorescences subtended by bracts on Arabidopsis leaves. Analysis of these novel leaf phenotypes revealed potential functions of UFO outside the flower context. Additionally, comparison of UFO functions with its orthologs in other plant species also suggests a broader role for UFO during meristem establishment and specification.

Results

UFO is a transcriptional co-activator

To assess the functions of UFO, Arabidopsis plants were transformed with the UFO gene under the control of the cauliflower mosaic virus 35S (CaMV 35S) promoter. 22% of the transgenic plants produced serrate leaves and flowers with reduced sepal and floral abnormalities in the development of the gynoecium valves and the style. Overall these plants displayed relatively milder phenotypes than the 35S:UFO phenotypes reported by Lee et al (Table S1; Figure 1B) [8]. Expression of UFO under the control of the LFY promoter also resulted in serrated leaves, but occurred less frequently among the T1 transgenic lines (4%) compared to the expression with the 35S promoter (22%), suggesting that higher UFO levels were also required for the overexpression phenotypes (Table S1). When UFO was over-expressed in the bg2-1 background, which suppresses gene silencing [25], all T1 plants showed the gain-of-function phenotypes with enhanced leaf serration (Table S1). These results suggest that the 35S:UFO plants (78%) with a weak and medium loss-of-function phenotypes in the wild type background were the result of partial silencing of the endogenous UFO gene and that high UFO levels were required for the observed leaf serration and other phenotypic changes (Table S1).

Flowers of the ask1-1 mutant showed fewer petals compared to wild type and were often replaced with petal-stamen chimeras, which resemble the flowers of an intermediate ufo-1 mutant implying a genetic interaction between these two genes (Figure 1C, D) [12,26]. Accordingly, the interaction of UFO with the most abundant ASK1 protein [18] was required for the 35S:UFO phenotype because the ask1-1 mutation was epistatic to the UFO transgene (Figure 1E) and over-expression of UFO carrying a mutation in the F-box resulted in a weak ufo dominant-negative phenotype (Figure 1F). These phenotypes are also consistent with the paradoxical “activation by destruction” model which has been implicated for LFY [10]. This model predicts that ubiquitination of transcription factors results in simultaneous activation and priming for destruction. Thus, UFO lacking the F-box domain is expected to stabilize un-ubiquitinated LFY and thereby repressing its activity. UFO is predominantly a nuclear protein as shown by the accumulation of a UFO-YFP fusion in the nucleus upon transient expression in Nicotiana benthamiana leaves (Figure 1G, H). This result also confirmed that translocation of UFO into the nucleus is required for its activity because fusion to the glucocorticoid receptor (GR), which is known to be retained in the cytosol in the absence of hormone, is unable to induce a gain-of-function phenotypes (Table S1; Figure 1 I, J). As expected, dexamethasone (DEX) induced translocation of UFO-GR from the cytosol into the nucleus, correlated with the initiation of serrated leaves and abnormal flowers in 23% of the transgenic lines (Table S1; Figure 1K, L). Taken together, the results showed that the function of UFO depends on its nuclear localization and its interaction with ASK1.

LFY is a key transcription factor in flower development and has been identified as a likely substrate for ubiquitination by UFO [10]. Because UFO does not contain any known DNA binding domains, we reasoned that UFO likely functions in the context of a transcriptional complex that includes LFY. In this situation, the fusion of UFO to the Drosophila Engrailed (En) transcriptional repressor domain or to the viral VP16 activator domain was expected to influence the function of this putative transcriptional complex in a dominant negative (loss-of-function) and a dominant (gain-of-function) manner respectively. This was indeed the case and the strong novel phenotypes identified in this study further indicate potential additional roles for UFO in plant development.

When the En-UFO translational fusion was over-expressed in Arabidopsis under the 35S promoter, ~85% of the T1 plants exhibited a range of mild to strong ufo-like phenotypes with normal leaves. Overall these phenotypes were stronger compared to the plants expressing UFO without the F-box, but weaker than the reported UFO-SRDX fusion (Table S1; Figure 1M) [10]. Upon combining the Engrailed domain with the F-box deletion, more T1 transgenic plants showed strong loss-of-function phenotypes with flowers containing sepal-petal, stamen-carpel chimeras or filaments in the second and third whorl or flower-filament substitutions in the inflorescence (Table S1; Figure 1N). In contrast, every T1 plant expressing the UFO-VP16 fusion exhibited leaf serration (Figure 1P, Q), which was more severe compared to plants over-expressing UFO only. RT-PCR analysis revealed an 8-fold increase in the UFO-VP16 transcript levels compared to endogenous UFO RNA consistent with the observed phenotypes (Figure S1; see Methods). Based on the onset and severity of the leaf serration, the transgenic lines could be divided into two groups. About 86% of the plants showed clear serration starting in the third or fourth true leaf and these were designated as the weak and medium phenotype group whereas the plants in the strong phenotype group showed serration of all rosette leaves (Table S1; Figure 1P, Q). Compared to wild type and 35S:UFO transgenic plants, the emergence of the first true leaves was delayed in UFO-VP16 expressing lines, especially in the plants that showed strong gain-of-function phenotypes. The serrations
became increasingly severe in later formed leaves. When UFO-VP16 was expressed in the gsg2-1 background, all T1 plants from the weak and medium phenotype group displayed a strong gain-of-function phenotype, indicating that the weaker phenotype was caused by either lower UFO-VP16 protein expression and/or by partial silencing of the endogenous UFO gene (Table S1; Figure 1R, S). The result that the VP16 transcriptional activator and not the En repressor enhanced the UFO over-expression phenotype suggests that UFO likely functions as a transcriptional co-activator.

**UFO-VP16 causes flowering leaves**

Overexpression of UFO fused to the VP16 transactivation domain resulted in unexpected phenotypes not observed in the earlier studies. 50% of the T1 plants, which belonged to the first group developed flowers along the adaxial edge beginning in the...
last three rosette leaves that are formed prior to bolting and later in all the cauline leaves (Figure 2A–C). These floral meristems were ectopic because they were neither derived from the apical meristem nor from the displacement of the axillary meristem. Although most of the leaf blade originated from the basal part of the leaf, the flowering rosette leaves often grew to a normal size. Sometimes the flowers were also subtended by a bract leaf (Figure 2D). Occasionally an inflorescence would develop in addition to the individual flowers (Figure 2E). These flowers were mostly male sterile, but produced viable seeds upon pollination (Figure 2F). Closer examination revealed that the lower rosette leaf primordia had serrations along their margins that became compounded at later stages as shown by secondary serrations (Figure 2G, H). These leaf primordia exhibited a prolonged primordial phase and eventually developed into severely lobed leaves that were covered with enlarged trichomes (Figure 2J). The upper rosette leaf primordia that were formed during floral transition were also serrated and initiated floral meristems that developed in a sequential acropetal fashion along the adaxial margins (Figure 2K–N). It is interesting to note that each developing floral meristem (indicated by red arrow in Figure 2M, N) was positioned at the distal end of a serration (indicated by yellow star in Figure 2M, N), and few of them formed bract-like structures (Figure 2D). These ectopic floral meristems followed the normal developmental progression as defined by the stages shown in the insets of Figures 2K–O–Q [27]. The floral meristems on the leaves developed into complete flowers including the pedicel, and were similar to the flowers produced on the main inflorescence. Often these ectopic floral meristems were fused to produce fasciations of the pedicel or of the flowers themselves. Few of these ectopic meristems developed into inflorescence meristems that produced flowers along their flanks (Figure 2M, 2E). These phenotypes indicate that the inflorescence program, which is normally established in the axis of the leaf, now expanded into the leaf primordium during its extended meristematic phase.

After the floral transition, the cauline leaves surrounding the apical inflorescence meristem also initiated ectopic floral meristems that covered most of the leaf blade (Figure 3A–D). These floral meristems were positioned in close proximity to the axillary co-inflorescences resulting in the fascination of the peduncle of the co-inflorescence and the petiole of the subtending flowering cauline leaf (Figure 3C, E). Similar to plants expressing UFO without VP16, flowers of the first group had mostly a normal organ number but the sepals were wrinkled and the petals, stamens and carpels were stunted (Figure 3F). However, the UPO-VP16 plants with a strong phenotype belonging to the second group exhibited a more disorganized inflorescence that was delayed in development (Figure 3G). The cauline leaf primordia initiated floral meristems as observed in the first group with weaker phenotype, but remained meristematic and failed to progress into normal flowers (Figures 3E, 3F bottom panel). The flowers on the primary inflorescence were clustered due to very short pedicels and delayed internodal elongation of the peduncle (Figure 3H, I). The flowers had serrated petal-sepal chimeric organs in the first whorl and the gynoecium was either incompletely fused or replaced by additional stamens (Figure 3J). Plants transformed with p35S:UFOdelF-VP16 that lacked a functional F-box domain failed to produce the above-described UPO-VP16 gain-of-function phenotypes and instead showed a loss-of-function phenotype similar to p35S:UFOdelF without VP16 (Table S1, Figure 3K). These results further confirmed that F-box domain of UFO is required for VP16 mediated gain-of-function phenotypes.

**Meristem specification by UFO in other plant species**

Transformation of the related crucifer *Brassica napus* with p35S:UFO-VP16 resulted in severe serration of the leaves in addition to the lobing observed in wild type leaves (Figure 4A–D, Table S1). These serrations were compounded as a result of reiterated serrations during leaf development (Figure 4D). Closer examination of a young leaf showed a highly meristematic leaf margin, which likely contributed to these progressively increased leaf serrations in the older leaves (Figure 4E, F). The sinus regions of these leaves often remained meristematic producing new projections for a prolonged period (Figure 4G inset). Though, expansion of the leaves was delayed, the size of the mature leaves often surpassed that of the wild type plants. Similar to Arabidopsis, the cauline leaf blade was consumed by proliferating ectopic floral structures (Figure 4G, H). The weaker p35S:UFO-VP16 lines showed meristematic cauline leaf margins without ectopic floral meristems (Figure 4H, J). The flowers produced by the primary inflorescence in the weaker lines were clustered without sufficient elongation of the internodes and with shorter pedicels whereas in the stronger lines, a mass of proliferating floral organs was produced (Figure 4G, H). Taken together, though some differences were observed, the p35S:UFO-VP16 mediated gain-of-function phenotypes of *B. napus* were comparable to that of Arabidopsis.

To determine whether UFO could induce similar phenotypes in more unrelated plant species, the UFO constructs were transformed into tobacco (*Nicotiana tabacum*). In the tobacco cymose inflorescence, the apical inflorescence meristem and the consecutive co-inflorescences terminate in a flower, whereas in the crucifers i.e., Arabidopsis and *B. napus*, the indeterminate racemose inflorescence produces flowers in an acropetal fashion. Tobacco plants over-expressing UFO produced light green sectors starting in the third leaf as well as more curling of the margins compared to wild type (Figure 5A, B; Table S1). The cellular organization of these light green sectors in the cross sections showed high similarity with sepal as opposed to leaves of wild type plants (Figure 5K–M). Pink pigmented sectors were also present occasionally in the upper leaves (Figure 5E), indicative of the chimeric nature of the vegetative leaves mixed with characteristics of sepal and petal cell types. The severity of the leaf phenotype correlated with earlier flowering, on average 57 days after seeding in p35S:UFO plants versus more than 90 days in wild type. The typical whorled phyllotaxy of wild type tobacco flowers was absent in the UFO over-expression lines (Figure 5F–I). Instead, the flowers exhibited a continuum of the spiral phyllotaxy of the shoot where the distinct floral whorls seen in wild type were replaced by a gradient of organ mosaics starting from cauline leaves that continued into sepals, sepal-petals, petals, petal-stamens and carpels (Figure 5F–H). The UPO-VP16 tobacco plants flowered very early (~41 days after seeding) after producing about 5 leaves (Figure 5C); leaf curling and mosaics started in the second leaf but no ectopic floral meristems developed (Figure 3J). Taken together, UFO overexpression in tobacco had a distinct phenotypic effect compared to that observed in *B. napus* and Arabidopsis, highlighting the dependence of ectopic UFO or UPO-VP16 on the existing developmental programs and the associated genetic factors in these species.

**UFO function depends on both LFY and SEP proteins**

Floral meristems of the *lfy-1* mutant default into a secondary co-florpuscence identity and are unable to develop a gain-of-function phenotype when UFO is overexpressed [7,8]. The dependence of UFO on LFY was also observed in the present study with UPO-VP16 as the *lfy-1* mutant was fully epistatic to the UPO-VP16
transgene (Figure 6A–C). Expression of the pLFY:GUS reporter in p35S:UFO-VP16 plants was similar to its expression in the wild type background (Figure 6D, E). Upon flowering, the GUS activity was strong in the shoot apex and leaf primordia of both lines, but was not detected in the emerging leaves. Also, LFY RNA levels of wild type and p35S:UFO-VP16 seedlings were similar as determined by RT-PCR (Figure S1). These results suggest that the LFY expression was not upregulated by UFO-VP16, and the observed phenotypes were more likely the result of a prolonged interaction between UFO-VP16 and LFY during the early stages of leaf development (see discussion).

Next we tested whether UFO-VP16 is involved in the regulation of the targets of LFY that include A, B, C and E class MADS-box genes [5]. Our results showed that pAPETALA1:GUS was expressed earlier in the lower leaves of UFO-VP16 seedlings prior to flowering, whereas pAPETALA3:GUS was detected only in the upper serrated leaves at flowering stage (Figure 6F, G). No ectopic expression was observed with the APETALA3, PISTILLATA and AGAMOUS promoters prior to flowering (not shown). These observations are consistent with the result that none of the ABC mutants were able to suppress the serrated leaf phenotype and the flowers on both the inflorescence and the leaves retained the characteristics from the non-transformed mutants. Interestingly, serration was enhanced in the ap1 mutant and the ectopic meristems on the leaves developed into ap1-like flowers (Figure 6H). These observations suggest that the ABC class MADS box genes were not required for the gain-of-function leaf phenotypes.

MADS-box proteins function in quaternary complexes to regulate the identity of the meristems and primordia in the above-ground region of the plant [28]. Generally at least one position in the complex is taken by a member of the four SEPALLATA MADS-box proteins (SEP1, SEP2, SEP3, SEP4), to mediate the formation of higher-order complexes [29]. Because the SEP proteins function in a partially redundant manner, the UFO-VP16 construct was transformed into fertile sep1/sep1,sep2/...
plants using the flower dip method and analyzed in the segregating offspring. The majority of 36 sep UFO-VP16 T1 plants (24 plants) did not show leaf serration, indicating that some combinations of the sep alleles had suppressed the gain-of-function phenotype, but instead showed flower phenotypes similar to the respective sep mutants without the transgene (Figure 6I–N). The second group (12 plants) showed various levels of serration, including 5 with ectopic flowers on the leaves and flowers typical for UFO-VP16 (Figure 6O–Q). Genotyping of the segregating plants for the 4 SEP genes (see methods) revealed that the sep mutant alleles in various combinations were sufficient to suppress the UFO-VP16 phenotype of the first group. All plants with one wild type SEP allele and seven out of eight plants with two SEP alleles belonged to the first group, showing a positive correlation between the UFO-VP16 phenotype and the presence of a higher number of wild type SEP alleles. Furthermore, all seven plants carrying both SEP4 alleles had serrated leaves including five carrying ectopic flowers, indicating that SEP4 was required and is more critical than the other SEP proteins for this gain-of-function leaf phenotype. RT-PCR analysis revealed no differences in SEP transcript levels between UFO-VP16 and wild type seedlings (Figure S1) suggesting that higher expression levels of the SEP genes were not required. Taken together, these results suggest that in addition to LFY, the SEP proteins were also required for UFO gain-of-function phenotypes in Arabidopsis.

To test whether over-activation of SEP could result in similar phenotypic changes as observed in UFO transgenic plants, VP16 based activation constructs were developed with SEP1 and SEP4. SEP4-VP16 over-expressing plants exhibited mild serration of the leaves, which often curled up at the edges (Table S1, Figure 6S). No ectopic meristems were observed on the leaves. However the flowers were strikingly similar to those observed in UFO-VP16 plants. The pedicels were short and the growth of the peduncle was delayed giving the inflorescence a compact appearance (Figure 6T). Further, the development of the flower buds was delayed for an extended period. Plants with a weak phenotype were taller with uneven internodal distances between the flowers. A similar weak phenotype was observed in plants transformed with

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Figure 3. Inflorescence phenotypes of p35S:UFO-VP16 Arabidopsis plants. (A–F) p35S:UFO-VP16 inflorescence with a medium phenotype showing the developing flowers (outlined in red) produced by the primary inflorescence meristem (red star) (A - top view, B - side view) and by the cauline leaves (blue star) shown at different developmental stages (A–E); the developing co-flowered meristem (green star) and the transformed cauline leaf primordium (C) fail to separate leading to a fasciated structure (E); flowers show reduced and short petals, stamens and carpels (F). (G–J) p35S:UFO-VP16 inflorescence with a strong phenotype showing an disordered inflorescence meristem (red star) with delayed development of the floral primordia (G) that eventually bolts to produce flowers (H) with serrated floral organs and short pedicles (I-top panel, J) and cauline leaves (blue star) with ectopic disordered floral meristems (G) that develop into abnormal flowers (I-bottom panel). (K) p35S:UFOdelFVp16 flowers with a medium ufo-1 like phenotype. Bar = 0.1 mm (A-D, G).

doi:10.1371/journal.pone.0083807.g003

Figure 4. Phenotypes of Brassica napus plants expressing p35S:UFO-VP16. Wild type plant (A) showing normal lobed leaves (B). (C–F) p35S:UFO-VP16 plant (C) showing severe lobing of the leaf (D); expanding young leaf (E) showing meristematic activity along the leaf margin (F) that leads to the enhanced lobing. Inset in (C) shows prolonged meristematic activity of the leaf margin. (G–J) Inflorescences of p35S:UFO-VP16 plants showing severe proliferation of floral organs produced by the inflorescence meristem (G) and by the cauline leaves (G, I; white arrows); weaker phenotype showing the development of modified flowers with short pedicels (H) and prolonged meristematic activity of the caulinle leaf margins (H, J).

doi:10.1371/journal.pone.0083807.g004
SEP1-VP16. Both transgenics flowered at normal times, which contrasted with the very early flowering of p35S:SEP1 transgenics and prematurely terminating in a terminal flower (Table S1, Figure 6R). Taken together, the results indicate that UFO functions as a co-activator in concert with LFY and prematurely terminating in a terminal flower (Table S1, Figure 6R). Taken together, the results indicate that UFO functions as a co-activator in concert with LFY and prematurely terminating in a terminal flower (Table S1, Figure 6R). Taken together, these phenotypes indicate that UFO-LFY transcriptional complex. Overexpression of SEP1 resulted in early transition of the apical meristem into a terminal flower similar to the phenotypes of plants overexpressing LFY or SEP3 [30,31], whereas SEP4-VP16 plants showed some phenotypic similarities with plants expressing UFO-VP16 in the flowers. These results are consistent with the study that showed physical interaction between LFY and SEP3 proteins, and further support the model that the UFO, LFY and SEP proteins most likely interact in a transcriptional complex to regulate a common set of target genes involved in meristem specification and flower development [31].

Our results also show that in addition to LFY, SEP proteins were required for the observed strong UFO-VP16 leaf serration phenotypes implying that SEP proteins may also associate with the UFO-LFY transcriptional complex. Overexpression of SEP1 resulted in early transition of the apical meristem into a terminal flower similar to the phenotypes of plants overexpressing LFY or SEP3 [30,31], whereas SEP4-VP16 plants showed some phenotypic similarities with plants expressing UFO-VP16 in the flowers. These results are consistent with the study that showed physical interaction between LFY and SEP3 proteins, and further support the model that the UFO, LFY and SEP proteins most likely interact in a transcriptional complex to regulate a common set of target genes involved in meristem specification and flower development [31].

Function of UFO outside the APETALA3 domain

UFO has been shown to bind the AP3 promoter in the presence of LFY to activate its expression in the stage 3 flower primordia [8,10]. In the ufo-1 mutant compared to wild type, AP3 expression is restricted to a narrower domain in stage 4 and in later stages of flower primordia [17]. When UFO is overexpressed, the vegetative leaves become serrated, suggesting that the leaf primordia are responsive to changes in UFO expression [8]. Previous studies have shown that ufo mutants display delayed transition of the vegetative to inflorescence identity of the apical meristem. Moreover, some ufo flowers also lack organs in the 1st and 4th whorls that are established before AP3 expression, whereas other flowers are substituted by filamentous structures [11,12]. In the ufo-1 mutant, AP1 expression is reduced in stage 1 and 2 flowers [32] and consistent with this, our results show ectopic AP1 expression in UFO-VP16 leaves. Taken together, these phenotypes indicate that UFO in the presence of LFY is also responsible for the activation of genes other than AP3.

It was previously shown that UFO expression is initiated very early in the embryonic shoot meristem by SHOOTMERISTEM-
LESSTM and is maintained in the peripheral zone (PZ) of the vegetative shoot (SAM) and inflorescence (IM) meristems throughout development [8,33,34] (Figure 7A). In the SAM, UFO expression overlaps with that of LFY in the domains of P0 and P1 stage leaf primordia, whose expression in turn is initiated by the auxin maximum through MONOPTEROS (MP), an ARF family member of the auxin signaling pathway [35,36]. LFY is expressed at very low levels in the first primary leaf primordia, but becomes increasingly stronger in the upper rosette and cauline leaf primordia [36]. In the later stages of leaf primordia, UFO expression is not detected whereas LFY continues to be expressed [34,36] (Figure 7A). In the IM, UFO and LFY expression domains overlap in stage 0 floral primordia. UFO is reactivated in the centre of stage 2 flower meristems after the establishment of the first sepal whorl. When AGAMOUS expression is initiated in the centre during early stage 3, UFO RNA is restricted to a cup-shaped domain between the first and fourth whorls followed by expression at the base of petals during stage 4 while the third whorl is established [8] (Figure 7A). Taken together, UFO expression is not restricted to the AP5 domain in the floral meristem, but overlaps with LFY during the early developmental stages of all lateral meristems/primordia suggesting an ancestral role in their establishment and fate specification.

UFO is involved in the establishment of meristems

Based on the native expression patterns of UFO and LFY, we propose two functional stages for LFY activity (Figure 7B). The earlier stage which involves UFO as a co-activator is important for the lateral meristem (primordia) establishment in the PZ of the SAM, whereas the later stage of LFY function, which does not involve UFO, is primarily to promote differentiation and organogenesis of the primordia. Over-expression of UFO or UFO-VP16 resulted in repeated lobing of the leaf primordia and delayed emergence in Arabidopsis (Figure 2G, H). In B. napus, the meristematic activity was continued in the margins of mature leaves expressing UFO-VP16 transgene. sep2-1 carries an En-1 insertion in the seventh intron, which has excised in the SEP2-1rev revertant allele. Flowers with bract-like organs (IJ) and a normal rosette (K); normal flowers (L,M) and rosette (N): normal flower (O) and UFO-VP16 like flowers that have short pedicels with a flowering cauline leaf (arrow, P); UFO-VP16 like rosette (Q). (R) p3SS:SEP1 inflorescence terminating in a flower. (S, T) p3SS:SEP4-VP16 rosette with leaves showing serration (S) and inflorescence producing flowers with short pedicels (T).
impaired, which is evident from the premature termination of the inflorescence meristem into a flower in strong ufo mutants. The milder ufo mutant phenotypes compared to those of lfy support the argument that UFO is not required for LFY functions in the later stages. Overexpression of LFY does not result in the production of serrated leaves with ectopic meristems, indicating that unlike UFO, increased LFY levels alone are not sufficient to extend the meristematic phase of the leaf primordia [30]. However, seedlings overexpressing the hyperactive LFY-VP16 form of LFY in combination with UFO lead to growth arrest, implying that higher LFY activity is important for lateral meristem establishment and that a regulated lower activity is necessary to promote growth and differentiation [9]. Overexpression of the F-box genes most similar to UFO (LEAF CURLING RESPONSIVENESS [LCR; At1g27340, At1g76920, At4g33160] with or without VP16 did not result in the production of aberrant phenotypes (not shown), whereas LCR has been implicated in the expansion of the leaf margin [40], suggesting that UFO does not function in a redundant manner with other F-box proteins.

UFO ensures the proper fating of meristems

Phytomers are produced by the apical meristem and their fates in turn determine the identity of the vegetative, inflorescence and floral meristems [1]. The identities of the apical and lateral meristems are determined by the combinatorial co-expression of specific MADS box proteins during development [41]. LFY is responsible for activation of the MADS box genes that specify the floral meristem (AP1, CAL, FUL) and floral organ identities (AP1, AP3, PI, AG, SEP) [42]. Overexpression of LFY results in precocious expression of these MADS box genes leading to early flowering by homeotic transformation of the lateral shoots into single flowers followed by the premature conversion of the primary shoot into a terminal flower [30]. These overexpression LFY phenotypes are not UFO dependent. However, overexpression of UFO-VP16 in the presence of wild type LFY results in ectopic flowers and inflorescences in the upper rosette and cauline leaves (Figure 2, 3). The arrangement of older floral meristems along the main leaf margins (Figure 2K, L) and the emergence of the new meristems in acropetal succession along the margins of the secondary leaf serrations (2L–N) are reminiscent of the acropetal development of flowers on the primary inflorescence. Furthermore, the positions of the secondary and tertiary serrations of the upper rosette leaves are indicative of a bract-like fate (Figure 2M, N) which becomes obvious in later stages of these ectopic inflorescences (Figure 2C, D). The source of the shoot-like phenotype of the leaves may originate in the axillary meristem. In Arabidopsis, immediately after emergence of the leaf primordium, a cell niche is specified in its axis for future development of the axillary meristems [43]. REGULATOR OF AXILLARY MERISTEMS (RAX1) functions to specify this axillary meristem niche and its expression is directly induced by LFY [44]. The fasciation of the co-inflorescences with the cauline leaves observed in UFO-VP16 suggests that the extended meristematic phase of the leaf primordia delays the boundary formation between the axillary

**Figure 7. Model depicting UFO activation of LFY and their roles in the early stages of lateral meristem development.** A. UFO (blue) is expressed in the peripheral zone of the shoot apical (SAM) and inflorescence (IM) meristems. UFO is later induced in the centre of stage 2 floral meristems and its expression domain is restricted to the second and third whorls during later stages [17,34]. LFY expression (shades of orange) is initiated in the P0 leaf primordium and progressively increases in the later stages; its expression becoming prominent when floral meristems are initiated. The UFO and LFY expression domains overlap in the lateral P0 and P1 leaf primordia in the SAM, in stage 0 floral primordia in the IM, and in the petal, stamen and carpel whorls upon establishment [8,36]. B. LFY activity is enhanced by UFO in the emerging lateral meristems/primordia within the peripheral zones of the meristems to ensure their establishment and fating. Lower LFY activity without UFO promotes the organogenesis of the primordia during later stages.

doi:10.1371/journal.pone.0083807.g007
meristems and the leaf primordia thus allowing the co-inflorescence meristem program to expand from the axis into the leaf primordia.

Contrastingly in the uf0 mutants, homeosis of the lateral meristems/primordia is the result of insufficient induction of AP3 and LFY [17,32], but may also apply to other genes induced by LFY [45]. In these situations, the primordia acquire an identity which was established earlier during development. For example in uf0 flowers, petals become sepaloid and stamens carpelloid, whereas flowers are replaced by co-inflorescences or subtended by bracts. Taken together the UFO overexpression and uf0 mutant phenotypes suggest that UFO in combination with LFT provides a framework for the meristems and organ primordia to establish the expression of the proper combination of identity genes, possibly by promoting the meristem fate and/or by suppressing downstream morphogenesis programs (Figure 7B).

**UFO functions in other plant species**

In Arabidopsis, flowers are produced in the lateral positions of the inflorescence meristem (raceme), whereas in tobacco, flowers are positioned at the terminus of the primary and co-inflorescences (cyme), which is a function of LFT and TERMINAL FLOWER1 (TFL1) expression in the IM [46]. Thus the flower identity in a racemose inflorescence is specified de novo in the lateral meristems, whereas in a cyme, the flowers are produced by a transition of the (co-) inflorescence fate into a floral identity. In tobacco, overexpression of UFO and UFO-VP16 result in early flowering due to the precocious and gradual transition of the apical vegetative meristem into a terminal flower. Consistent with this interpretation, mutants of UFO orthologs DOUBLE TOP (DOT) and ANANTHA (AN) in the related Solanaceae species petunia and tomato, respectively, are unable to transit into a flower and instead reiterate the inflorescence program. The LFT orthology NFL1 (tobacco), ABEROANT LEAF AND FLOWER (ALF) petunia) and FALSEFLORA (F1) tomato) are expressed like LFT in the lateral meristems produced by the vegetative and inflorescence meristems, but the NFL1 transcript levels are higher than those of LFT during the vegetative stage [39,47,48]. The UFO orthologs DOT in petunia and AN in tomato are not expressed in the vegetative meristems, but are first activated in the IMs upon transition to flowering [15,49]. Thus in the cyme inflorescence, DOT/AN most likely plays an essential role to establish the floral identity in the apical meristem by activating the LFT orthologs.

The serrated leaves in UFO overexpressing Arabidopsis plants display some common features with UFO/LFY orthologs in compound leaf development in other plant species. In the compound leaves of tomato the number of leaflets is slightly reduced in the f0 mutant [39], although the majority of the leaf form is controlled by the KNOX gene pathway [50]. However, the pea STP/UNI pathway is important for the initiation of pinnae (i.e. leaflet and tendril) primordia in the compound leaves. stp (UFO ortholog) mutants have reduced numbers of pinnae and uni (LFT ortholog) plants have trilobate or simple leaves [38]. Both of these mutants exhibit a prolonged meristematic phase while initiating lateral primordia in an acropetal manner and the process is reiterated in the lateral primordia upon ectopic expression of the LFT/UNI and/or UFO/STP genes [16]. Both STP and UNI are expressed in early leaf primordia and UNI retains expression in the distal region of the marginal blastozone until the apex terminates in a distal tendril. In the aitl and cachita mutants UNI is also expressed in the pinnae and stipule primordia respectively, and results in the development of secondary rachis formation in these positions demonstrating that UNI promotes the rachis identity of the leaf [16]. These observations raise an interesting possibility that the underpinning genetic network which increases the size of the pea leaf by derepressing UNI, is similar to the reduced determinacy of the Arabidopsis leaves upon activation of LFT by overexpressing UFO. Moreover, the rachis specification of the leaf primordia by UNI and STP seems to operate in a similar fashion as the specification of the floral meristems in the inflorescence by LFY and UFO in Arabidopsis. More studies are required to address how LFT and UFO in combination with other genetic factors could produce different developmental outcomes in diverse plant species. In conclusion, we propose that the UFO orthologs play an ancestral role in activating LFY orthologs in the peripheral zone of the apical and floral meristems to promote the establishment and identity of the lateral meristems and primordia.

**Materials and Methods**

**Plasmid construction**

A new multiple cloning site including a C-terminal E-tag (GTGTTAAGCCACTAGTAAAGATCTAAGATTGTTCGCCGGGTCCGCTATCCGAGATC-GCGTGAACCGCGGTCTCGAGATCGAGCTTCTAGA) was generated by overlapping primers and cloned in the Pmda and Xhol sites of the binary Gateway destination vector pK7WG2 (VIB-Ghent University). The t35S terminator was PCR amplified with primers CACCTCGGATGACGGCCATGCTAGAGTGATGGATCTAGAGCTCGCCA and TCTAGAGTACCTGGATTGTTGGTTAGG, and cloned as an Nhel/Xbal fragment in the respective sites of the new MCS. The Br/G1 Gateway cassette (GW) fragment and the Pmda/Xpol p35S promoter fragment were reintroduced from pK7WG2 and cloned in the respective sites of the new MCS resulting in pER310. The engrapsed reverse domain was PCR amplified from pLD16125 (Drosophila Genomics Research Centre) with primers CACCAACTAGTATGCGCCCTTGGAG-TAGCCTGCG and AGATCTGAGATCCAGACAGATTTCTC and inserted at the N-terminal side of the GW site in pER310 resulting in pER311. The glucocorticoid receptor was amplified with primers CACCCCTGAGCAAGAAAAAAATCAAAGGGATTG and TCGGCGATCTATTTTGTAGAAGAAG and cloned in frame downstream from the E-tag in pER310 leading to pER312. Likewise in pER340 the VP16 domain was inserted after amplification with primers CACCGCTCGAGCCGGGGGCATGTCGACTGCTGATCGCGATCATCACCCAGTTGCTCGTACGCAAT. YFP was amplified with primers CACCACTAGTCTGGATATTATGGTACGAGAAGGGGC-GAGGA and TCGGCGATTGAGATCCAGACAGATTTCTC and cloned in the respective sites of the Gateway cassette (GW) fragment and the Pmda/Xpol p35S promoter fragment were reintroduced from pK7WG2 and cloned in frame downstream from the E-tag in pER310 resulting in pER356. The coding sequences without stop codon of UFO, UFOdelF (codons 30–62 deleted), SEP1, 2, 3, 4, and GUS were cloned in pDONR201 (Invitrogen) before recombination in the designated T-DNA destination vectors. The three-way pER557 was generated by substituting the p35S promoter and attR1 fragment of pER310 with the attR4 recombination site. The promoters including the start codon of the Arabidopsis genes LFY (-2321), AP1 (-1455), AP3 (-1240), PI (-960) and AG (-563-codon 83) were cloned into pDONR PI-P1R (invitrogen) and combined with GUS or other genes in pER557 using LR clonase.

**Plant materials, transformation and genotyping**

Mutants used in this study: ap2-1 [25], ap1,2,3,4 [51], fp1-1, uf0-1, asl1-1, asp1-10, po-1, ap3-1 and ag were provided by the Arabidopsis Biological Resource Center (ABRC, Ohio). Plants were grown at 22°C, 4000 lux under long day (16 h light) or short day (10 h light) regimes. Constructs were transformed in wild type Arabidopsis thaliana ecotype Columbia or in mutants with their
designed background using the Agrobacterium floral dip method [52]. Transgenic plants were selected on ½ MS medium supplemented with 10 g/L sucrose and 10 mg/L L-PPT or 50 mg/L kanamycin, and transplanted in soil after seven days. UFO-GR plants were induced by spraying with 30 µM dexa-methasone, 0.01% Tween-20 in water. Genotyping of the SEP alleles was performed as described by Ditta et al. [51]. Brassica napus DH1250 hypocotyls and Nicotiana tabacum Xanthi leaf segments were used for Agrobacterium mediated transformation. The number of transgenic lines analyzed in this study is summarized in Table S1. Flowering time of the transgenic tobacco lines was compared with wild type after seeding T1 seedlings of 35S:UFO-VP16 and Col WT using the Applied Biosystem Step One real-time PCR system and the SYBR Green PCR master mix as detailed in [53]. The tissue used for qRT-PCR included the SAM and surrounding primary leaves. The primers used for qRT-PCR are listed in Table S2.

qRT-PCR
The UFO, LFY, SEP1, 2, and 3 transcript levels and relative expression levels were quantified by qRT-PCR using actin 2 as internal controls using the methods described in [53]. The qRT-PCR experiment was performed in triplicate on 5 pools of 10-day-old T1 seedlings of 35S:UFO-VP16 and Col WT using the Applied Biosystem Step One real-time PCR system and the SYBR Green PCR master mix as detailed in [53]. The tissue used for qRT-PCR included the SAM and surrounding primary leaves. The primers used for qRT-PCR are listed in Table S2.

GUS staining and microscopy
Plant tissue was vacuum infiltrated in a solution containing 100 mM NaHPO4 pH 7.0, 10 mM Na-EDTA, 5 mM Na-ferricyanide, 5 mM K-ferrocyanide, 0.1% Triton X-100, 1 mg/ml 5-bromo-4 chloro 3 indolyl β-D-glucuronide (X-Gluc) and incubated overnight at 37°C. Tissue was cleared in 95% ethanol. Plant samples for scanning electron microscopy (SEM) were fixed overnight in 3% glutaraldehyde in 25 mM NaHPO4 buffer pH 7.0 at 4°C. After rinsing with the same phosphate buffer, samples were transferred to PO4 buffer with 1% OsO4 and incubated for 2 hours at room temperature. After rinsing with buffer the samples were transferred to 100% acetone with 10% increments. After two more changes with acetone the samples were critical point dried, mounted on aluminum stubs and coated with gold for 3 minutes in an Edwards S150B sputter coater. The samples were observed under a Phillips 505 scanning electron microscope at 30 kV and images were captured on Polaroid film. Images were scanned and edited in Adobe Photoshop CS (Adobe Systems, San Jose, California) to improve the contrast and place scale bars.

Supporting Information
Figure S1 RT-PCR results of gene expression levels in p35S:UFO-VP16 seedlings compared to wild type Arabidopsis. (TIF)
Table S1 Summary of UFO transgenic lines used in this study showing the distribution of the various phenotypes. (DOCX)
Table S2 Primers used for RT-PCR analysis. (DOCX)

Acknowledgments
We thank Shelley Hepworth and the two anonymous reviewers for their critical suggestions and helpful comments. National Research Council publication number 50141.

Author Contributions
Conceived and designed the experiments: ER PV RD. Performed the experiments: ER PV DN VB. Analyzed the data: ER PV RD. Contributed reagents/materials/analysis tools: WC KK TD. Wrote the paper: ER PV RD.

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