A novel allele of FILAMENTOUS FLOWER reveals new insights on the link between inflorescence and floral meristem organization and flower morphogenesis

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Abstract
Background: The Arabidopsis FILAMENTOUS FLOWER (FIL) gene encodes a YABBY (YAB) family putative transcription factor that has been implicated in specifying abaxial cell identities and thus regulating organ polarity of lateral organs. In contrast to double mutants of fil and other YAB genes, fil single mutants display mainly floral and inflorescence morphological defects that do not reflect merely a loss of abaxial identity. Recently, FIL and other YABs have been shown to regulate meristem organization in a non-cell-autonomous manner. In a screen for new mutations affecting floral organ morphology and development, we have identified a novel allele of FIL, fil-9 and characterized its floral and meristem phenotypes.

Results: The fil-9 mutation results in highly variable disruptions in floral organ numbers and size, partial homeotic transformations, and in defective inflorescence organization. Examination of meristems indicates that both fil-9 inflorescence and floral meristems are enlarged as a result of an increase in cell number, and deformed. Furthermore, primordia emergence from these meristems is disrupted such that several primordia arise simultaneously instead of sequentially. Many of the organs produced by the inflorescence meristems are filamentous, yet they are not considered by the plant as flowers. The severity of both floral organs and meristem phenotypes is increased acropetally and in higher growth temperature.

Conclusions: Detailed analysis following the development of fil-9 inflorescence and flowers throughout flower development enabled the drawing of a causal link between multiple traits of fil-9 phenotypes. The study reinforces the suggested role of FIL in meristem organization. The loss of spatial and temporal organization of fil-9 inflorescence and floral meristems presumably leads to disrupted cell allocation to developing floral organs and to a blurring of organ whorl boundaries. This disruption is reflected in morphological and organ identity aberrations of fil-9 floral organs and in the production of filamentous organs that are not perceived as flowers. Here, we show the role of FIL in reproductive meristem development and emphasize the potential of using fil mutants to study meristem organization and the related effects on flower morphogenesis.

Background
The shoot apical meristem (SAM) consists of a relatively small population of pluripotent cells, proliferation and allocation of which gives rise to the entire above ground plant body. The SAM is organized into three zones, namely a central zone (CZ) at the SAM summit in which cells divide slowly to replenish the SAM population, a slowly-dividing organizing center (OC) which lies below the CZ, and a peripheral zone (PZ) in which cells divide more rapidly and become allocated to presumptive organ primordia [1]. SAM homeostasis is dependent on the appropriate balance of cell proliferation and differentiation among these three zones.

These meristematic domains are maintained in part by a negative feedback loop between WUSCHEL (WUS) and CLAVATA3 (CLV3) (for recent reviews, see [2-4]). WUS
encodes a homeodomain-containing transcription factor that is expressed in the OC and acts to promote the expression of the CLV3 ligand in the CZ [5]. CLV3 is thought to bind to the CLV1/CLV2 receptor complex, which in turn limits the domain of expression of WUS [6]. The indeterminate state of cells within the meristem is also dependent on members of the KNOX gene family that are expressed in the SAM but excluded from incipient organ primordia [7].

Cells at the periphery of the CZ begin to differentiate concomitant with the downregulation of KNOX genes and the expression of AINTEGUMENTA (ANT), regulating cell proliferation in the emerging organ primordia [8,9]. Organ outgrowth is accompanied by the establishment of a boundary zone separating the primordium from the adjacent meristematic tissues [2]. Lateral organ polarity is determined by the converse activities of abaxially-expressed KANADI and members of the YABBY (YAB) gene family and of the adaxially-expressed PHABULOSA and PHAVOLUTA genes [10]. SAM activity is also regulated by signals emanating from the organ primordia. Several studies have demonstrated that abaxialization of organs by ectopic expression of abaxial genes or by repression of adaxial genes causes arrest or loss of the SAM [10-13]. Additionally, in Petunia, the Hairy Meristem gene that is expressed in organ primordia is required for SAM maintenance [14].

Upon transition from the vegetative to the reproductive phase, the SAM becomes an inflorescence meristem (IM). The IM gives rise to lateral meristems in its PZ which acquire a floral fate to become floral meristems (FMs). The FMs differentiate into flowers composed of four whorls of different types of floral organs. In Arabidopsis, specific combinations of the four classes of organ identity genes (ABCE) specify the type of organ formed in each flower whorl. The ABCE factors are expressed specifically in the whorls of their function, with spatial specificity of their expression domains being critical for correct floral organ differentiation and avoiding the appearance of chimeric organs with mixed identities [15].

Proper flower development demands maintenance of the borders between the whorls within the flower and between the organs in each whorl. A and C class factors negatively regulate each other, thereby creating mutually exclusive expression domains divided at the second and third whorl boundary. SUPERMAN (SUP) regulates the border between the third and forth whorls, preventing expansion of B factors into the forth whorl [16]. UNSUAL FLORAL ORGANS (UFO) activates the B class genes found in the second and third whorls and is thought to set up the inner and outer boundaries of the B domain [17,18]. CLIP SHAPED COTYLEDON1/2 regulate the separation of organs within whorls (sepalas and stamens) [19,20] and are also expressed in the borders of whorl 2, where they inhibit proliferation of cells in these regions [21].

In a mutant screen for plants affected in floral organ development, we have isolated a new allele of *filamentous flower* (*fil*), a member of the YAB (YAB) gene family. The YAB genes, FILAMENTOUS FLOWER (*FIL*), YAB2 and YAB3, are all expressed on the abaxial side of developing vegetative lateral organ primordia [12,22-24]. *FIL* is expressed in a dynamic fashion during floral development, with expression initially seen throughout the abaxial side of floral meristems, and later on the abaxial sides of floral organ primordia [22]. *fil yab3* double mutants exhibit a loss of adaxial-abaxial polarity in the vegetative organs and occasional formation of ectopic SAM structures on leaves [12]. Yet, *fil* single mutants do not show a vegetative phenotype [12,25-27] but rather are strongly affected in flower structure and floral organ number, morphology and to some extent, identity.

*fil* mutant plants also show abnormalities in inflorescence development. As *fil* floral phenotypes can not be explained in terms of loss of abaxial identity, a different role for *FIL* in inflorescence and flower development is likely. Earlier studies, in addressing the range of *fil* phenotypes, had indicated that *FIL* plays multiple roles in inflorescence and flower formation and development [25-28]. Recently, it was suggested that *FIL*, together with YAB3 and other genes, regulates the organized growth of the SAM via a non-cell-autonomous mechanism [24,29]. By driving expression of YAB family members in the PZ of the SAM, alterations in the expression of CZ markers could be detected, implying that YAB genes function in signaling across the meristem [24]. *FIL* itself is redundantly regulated by the myb domain gene, ASYMMETRIC LEAVES (*AS1*), and the trans-acting siRNA gene, TAS3, both of which are expressed in organ primordia [30]. In turn, genes expressed in the meristem also control *FIL* expression [23].

In this study, we have isolated a new *fil* allele, *fil-9*, and dissected the process of reproductive organ development in the mutant. By following flower development from the inflorescence and floral meristems, we have discovered new features of *fil* phenotypes. We observe a correlation between the meristem phenotypes to different aspects of *fil* floral mutants. As such, we suggest a causal link between the role of *fil* in meristem organization and the multifaceted floral and inflorescence abnormalities.

**Results**

**Identification of a novel *fil* allele**

To identify new mutations affecting floral organ morphology and development, we screened a population of activation tagged T-DNA lines [31]. This screen yielded a mutant whose phenotype resembled that of *fil* loss-of-function plants [25-27]. When crossed with *fil-8* [27,32]
homozygous plants, all F1 progeny showed the fil mutant phenotype, indicating that the newly isolated mutant was allelic to fil. Therefore the novel mutant was named fil-9. fil-9 backcrosses yielded a 3:1 ratio of wild type versus fil mutant phenotypes in the F2 generation (92 wild type:29 mutant), indicating that the fil-9 mutation segregated as a single locus recessive mutation. In addition, the fil-9 line was outcrossed for three generations to eliminate any possible additional mutations.

The T-DNA insert was mapped to the third intron of the FIL gene (Figure 1A), using T-DNA- and gene-specific primers (see Methods). RT-PCR analyses using gene-specific primers (Figure 1A) spanning the region upstream to the T-DNA insert or the region downstream to the insertion were performed to verify that the T-DNA insertion indeed disrupts FIL gene expression. As demonstrated by RT-PCR, a partial transcript was produced, albeit at a somewhat lower level than in wild type. The full length transcript was not, however, detected (Figure 1C). The FIL region upstream to the T-DNA insertion contains the coding sequence of the zinc finger domain of the protein (amino acid residues 22-60; [26] as well as the putative self-interaction domain (amino acid residues 22-60; [33] but lacks the HMG-box DNA binding domain (amino acid residues 146-179; [26]; Figure 1B). Although a partial FIL transcript is produced by the fil-9 allele, the mutant phenotypes indicate that the T-DNA insertion most likely results in FIL loss-of-function and does not cause a dominant negative effect. Finally, it should be noted that fil-9 is in the Columbia-7 (Col-7) background [31], while all other characterized fil alleles are in the Landsberg erecta (Ler) background [12].

fil-9 affects floral form

Plants homozygous for fil-9 generated flowers with a highly complex and variable phenotype (Figure 2), such that each flower presented a different combination of organ types. These phenotypes consisted of alterations in organ number, morphology, and position (summarized in Table 1). It is noteworthy that most aspects of the mutant phenotype were more severe in later-arising flowers (i.e. flower 11 and later) than in early flowers (i.e. flowers 1-10). The number of sepals in the first whorl of many fil-9 flowers was either increased (five to seven sepals instead of four, Figure 2B) or decreased. Some of the later-arising flowers contained split sepals that remained fused at the base (Figure 2B, arrow). Other morphological defects included small sepals (Figure 2C), narrow and inwardly curled sepals (Figure 2D), and rarely, sepal-like blades on a narrow-filamentous base (Figure 2E).

The phenotype of the second whorl was most apparent, since reduction in petal number was noted from the first flowers. The first few flowers usually developed one or two petals (Figures 2B and 2C), while most late-arising flowers lacked petals (Figures 2D and 2E). Many of the petals were shorter or narrower than were wild type petals, and some had a filamentous base or were completely filamentous (Figures 2F-2I). In contrast to the general reduction in petal size, petals wider than those seen in the wild type also developed, albeit rarely (Figure 2O).

Functional stamens (i.e. pollen-producing) developed almost only in early-arising flowers (i.e. within the first five flowers), with the number of stamens being highly variable in fil-9 flowers. Determining the number of stamens was, however, difficult. Many filamentous structures that developed were most likely stamens in origin. However, it was often unclear from which whorl the filaments originated (Figures 2G-2I). Some of the stamens developed an anther presenting normal morphology yet that did not mature to shed pollen. Many of the stamens bore a distorted anther which could range from containing enlarged and swollen locules (Figure 2J, arrow) to assuming a flattened, elongated and curled structure (Figure 2K, arrowhead). Often, only a filament developed, with its edge being sometimes swollen and bent. Occasionally, stamens developed at the base of, or attached to, a petal (Figure 2H) or a sepal (Figure 2I).

Most fil-9 flowers developed normal carpels, yet only the first few flowers were fertile and produced seeds. Manual pollination with wild type pollen was successful, indicating that the semi-sterility of the plants resulted from the presence of mostly dysfunctional stamens. Nonetheless, in later-arising flowers, some carpels were not fully fused and the septum was not fully united (Figure 2K), some contained bent carpels, and in some, the style was elongated (Figure 2N). In a few cases, the gynoecium comprised three carpels. Very late flowers (i.e. starting approximately from the 35th flower) were composed only of a gynoecium, which was highly twisted (Figure 2L). This appearance corresponds to a characteristic termination form of Arabidopsis plants that fail to develop seeds [34].

fil-9 flowers thus showed a general reduction in organ number and size. However, the number of sepals and, occasionally, carpels was increased, while different organ types were occasionally enlarged, in comparison to wild type organs.

fil-9 floral organ identity defects

fil-9 flowers displayed an array of partial homeotic transformations in sepals, petals and stamens (Table 2). However, homeotic transformations occurred in less than a quarter of all flowers. Notably, these transformations were never complete, instead resulting in chimeric organs with mixed identity, with our definition of a chimeric organ not being based on the organ shape (e.g. a filamentous base). Instead, we considered an organ to have a chimeric identity only if it displayed tissue-characteristics of...
different organ types, i.e. sepals with characteristics of petal epidermis, etc. The most common transformed organs were petaloid sepals (usually sepals with white petaloid margins; Figure 2N, arrowhead). Very rarely, sepals with stigmatic tissue at the edge (i.e. carpeloid sepals) were observed (data not shown). Petals or petaloid organs developing locules (stamenoid petals) were also relatively frequent (Figure 2N). Much rarer were sepaloid petals (petaloid organs in the second whorl with a green-sepaloid tip; Figure 2O, arrow) or sepaloid organs con-
Figure 2. *fil-9* flowers show a wide range of organ morphological and identity defects. (A) Arabidopsis wild type flower. (B to U) *fil-9* mutant flowers (B to K and N to U, see text for detailed description), terminating inflorescence (L) and flowers along the inflorescence stem (M). Arrow in B indicates a split sepal, in E, a sepal with a filamentous base, in F, a short petal, and in G a narrow petal. Arrow and arrowhead indicate, respectively: A narrow petal and a stamen fused at the base to a sepaloid petal in H, filaments bearing a petaloid blade and a stamen fused at the base to a sepaloid petal in I, an enlarged, swollen anther and a flattened bent anther in J, a sepal with petaloid margins and a stamenoid petal in N, an enlarged petal and a sepaloid petal in O, a petal with a filamentous base and a sepaloid organ containing a stamen-like locule in P, and a filament topped by stigmatic tissue and a bract subtending a flower in Q. (R) A filament harboring a mosaic organ composed of an anther and a petal. (S) The cells of the petaloid tissue (shown by the box in R) are the conical cells that are characteristic of the petal epidermis. (T) The organ, surrounded by a box and seen in a higher magnification in U, resembles a stamen filament bearing stigmatic tissue at the edge. J, K and R to U are scanning electron micrographs.
Occasionally, fil-9 flowers contained stamens in which the anther elongated into a distal structure composed of conical petal epidermis cells (Figures 2R and 2S), or stamen-like filaments harboring stigmatic tissue (Figures 2T and 2U).

Overall, the changes in identity of fil-9 floral organs were variable and did not implicate simple reduction or ectopic expression of a specific class of organ identity genes. The partial transformation of sepals to petals suggested an expansion of B class function, while the formation of stigmatic papillae on stamen-like organs was indicative of a loss of B class activity. Transformation of petals into a more stamenoid structure and the development of locules in petals implied the expansion of C class function. On the other hand, some of the mosaic organs appeared to be stamens transformed into petals indicative of a loss of C class function. Thus, it appears that these partial homeotic transformations are the result of variable shifts in the domains of organ identity gene activity.

In addition to the marked floral defects, fil-9 plants showed other abnormalities reflecting defects in inflorescence development. These include elongated pedicels, the occasional development of a filamentous bract subtending the flower (Figure 2Q), and the clustering of flowers in relatively short segments along the inflorescence.
cence, separated by segments harboring short green filaments (Figure 2M).

The variable phenotype of fil-9 flowers suggested that the severity of the effect of the mutation was somewhat stochastic. The possibility that growth temperature could have an effect on the severity of mutant phenotype was thus tested. When fil-9 plants were grown at a higher temperature (i.e. 28°C versus 22°C), severe organ defects appeared in earlier-arising flowers, as compared to the wild type. In addition, the percentage of flowers containing organs with mixed identity was higher at 28°C (25%), as compared to the percentage obtained at 22°C (19%).

fil-9 morphological defects can be traced to the inflorescence meristem

The overall reduced number and size of fil-9 floral organs raised the possibility that these defects were caused by changes in meristem size. Therefore, fil-9 inflorescence apices were compared to those of wild type plants by SEM (Figure 3, Table 3). IMs from two developmental stages were analyzed. Early meristems were defined as those containing only two mature open flowers (Figures 3A-3C and 3F-3H), while late meristems were defined as those that had experienced the maturation of twenty flowers (Figures 3I-K).

IM size was significantly larger in fil-9 plants (Figures 3B, 3C, 3G, 3H, 3J and 3K), as compared to the Col-7 wild type (Figures 3A, 3F, 3I, Table 3). Interestingly, in both fil-9 and wild type plants, early meristems were significantly larger than were late meristems (Table 3). A similar pattern also appeared in another fil allele, i.e. fil-5, in the Ler ecotype background (Table 3). fil-9 IMs were deformed, with the early meristems being flatter than in the wild type (Figures 3G, 3H compared to 3F). Late fil-9 meristems were variable in shape, being either flatter or more swollen than in the wild type (Figures 3), 3K compared to 3I).

As was shown for the floral phenotype, the sensitivity of fil-9 plants to higher temperature was also reflected in an increase of IM size at a growth temperature of 28°C, as compared to growth at 22°C (Table 3). Wild type IMs displayed no sensitivity to high temperature (Table 3).

To test whether fil-9 FMs also differed from those of wild type plants, the size of the central zone, defined as the length between opposite sepals, was measured in flowers from both early and late inflorescences. In both cases, fil-9 FMs were significantly larger, as compared to those of wild-type plants (Table 4).

The increased size of fil-9 meristems could result from either an increase in cell number or cell size. To distinguish between these two possibilities, the number of cells in an area of 966 μm² was counted (an area corresponding to a circle engulfing approximately the whole wild type meristem; Figures 3L-3N). To facilitate the measurements, the outline of the cells in the meristem was marked by staining the plasma membranes with the lipophilic dye, FM-64, (red) [35] and by L1 layer expression of GFP fused to the plasma-membrane-localized PIN1 protein (green) [36]. The average number of cells in the defined meristematic area did not differ between the fil-9 and wild type plants (Figures 3L and 3M), although the fil-9 meristem is larger than the wild type. This indicates that the aberrancy of fil-9 meristems is not caused by larger cells but rather results from accumulation of more cells in the meristem, as compared to the wild type.

The enlarged fil-9 IM and FM are functionally disrupted. This is reflected in the irregular temporal order of emergence of floral primordia on the flanks of the IM, with several floral primordia arising simultaneously, instead of sequentially (Figures 3H (fil-9) and 3F (wild type), respectively). In addition, many floral organs emerge concurrently, both within the same and in different whorls within the flower (Figures 3B and 3C).

### Table 2: Organ Identity defects in fil-9

| Organ | Phenotype   | Percentage of flowers (%) |
|-------|-------------|---------------------------|
|       | Early flowers (1-10)<sup>a</sup> | Late flowers (≥ 11)<sup>b</sup> | Total<sup>c</sup> |
| Sepals | Petaloid | 17 | 25 | 22 |
|        | Carpeloid | 0 | 1 | 0 |
| Petals | Stamenoid | 11 | 19 | 16 |
|        | Sepaloid | 4 | 1 | 2 |
| Stamens | Carpeloid | 3 | 0 | 2 |
|        | Petaloid | 0 | 2 | 1 |

<sup>a</sup> Number of flowers scored was 70 (7 plants).
<sup>b</sup> Number of flowers scored was 162 (7 plants).
<sup>c</sup> Number of flowers scored was 232 (7 plants).
Figure 3 fil-9 versus wild type inflorescence meristems and floral primordia. (A to C) Top view of early wild type (A) and fil-9 (B, C) inflorescences. The scale bar represents 100 μm. (D and E) A schematic representation of floral orientation relative to the IM in wild type and fil-9 plants, respectively. IM, inflorescence meristem; Se, sepal. (F to H) Enlarged view of early wild type (F) and fil-9 (G, H) inflorescences. Floral primordia are labeled 0, 1, 2, etc., in order of increasing age. The scale bar represents 20 μM. (I to K) Enlarged view of late wild type (I) and fil-9 (J, K) inflorescences. The scale bar represents 20 μM. IM, inflorescence meristem. (L to N) Top view of early wild type (L) and fil-9 (M and enlarged view in N) inflorescences, labeled with FM4-64 (red) PIN1-GFP (green). The scale bar represents 20 μM. The yellow circle designates the area used for cell number count. The average number of cells in the defined meristematic area did not differ between fil-9 and wild type plants (ANOVA, $F_{1,38} = 0.326, P = 0.571$).
An additional feature of floral emergence from fil-9 IM was the distorted orientation of the flowers towards the axis of the IM, as deduced by the position of the sepals (Figures 3B, 3C and 3E (fil-9) compared to 3A and 3D (wild type)). This was accompanied by changes in the order of sepal development. In wild type flowers, the abaxial sepal developed first, followed by the adaxial sepal and finally, the two lateral sepals (Figures 3A and 3D; [37]). However, in fil-9 flowers, two sepals, or other organs occupying that position, developed in abaxial lateral positions, with the two other sepals developing later, in the adaxial position (Figures 3B, 3C and 3E).

The filamentous structures produced by fil-9 inflorescence meristem do not replace true flowers

As previously noted, fil-9 defective inflorescence meristems produce several organs simultaneously, with many of these being filamentous organs with no clear identity. Earlier studies on other fil alleles reported that the development of such filaments is confined to a defined region along the inflorescence stem [25,26]. Under our growth conditions, we could not find filaments concentrated and/or limited to a specific zone, neither in fil-9 nor in two additional alleles (i.e. fil-5 and fil-8). To assess whether the filaments produced by the fil-9 IM have a floral identity, the number of flowers produced by the primary IMs during the course of reproductive development was compared between fil-9 and wild type plants. In these measurements, only organs with floral structures but not filaments were counted as flowers. During the first three weeks of flower production, fil-9 and wild type IMs produced the same number of flowers, at the same rate (Figure 4). However, in the fourth week, where as the wild type production rate did not change, fil-9 floral production accelerated (Figure 4). During the fifth week, wild type floral production sharply declined, while the rate of fil-9 floral production was only slightly reduced (Figure 4). Wild type flower production ceased after the fifth week, producing a total of 35 flowers on average, while fil-9 IMs proliferated for an additional week, producing a total of 60 flowers on average (Figure 4). This pattern of the proliferative capacity of fil-9 IM is similar to that of other reduced fertility mutants [34]. Thus, the production of more flowers by fil-9 IMs likely results from the partial self-sterility of fil-9 plants and not because of simultaneous emergence of more flowers from the IM. Moreover, the production of the same number of flowers, not including filamentous organs, in fil-9 as in wild type

Table 3: The average size of inflorescence meristem

|            | 22°C Early meristem mean ± SE (μm) | 28°C Early meristem mean ± SE (μm) | 22°C Late meristem mean ± SE (μm) | 28°C Late meristem mean ± SE (μm) |
|------------|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|
| Col-7      | n = 9                             | 52 ± 1.7                          | n = 9                             | 36 ± 1.8                          |
| fil-9      | n = 9                             | 77 ± 3.1                          | n = 9                             | 61 ± 2.9                          |
| Ler        | n = 7                             | 74 ± 2                            | n = 7                             | 45 ± 2.3                          |
| fil-5      | n = 8                             | 102 ± 3.1                         | n = 3                             | 52 ± 1.7                          |

Summary statistics

Col-7 vs. fil-9: ANOVA, F1,55 = 262.6, P < 0.001
Col-7: Early vs. Late meristems: ANOVA, F1,31 = 81, P < 0.001
Col-7: temperature (22°C vs. 28°C): ANOVA, F1,31 = 1.75, P = 0.195
fil-9: Early vs. Late meristems: ANOVA, F1,28 = 42, P < 0.001
fil-9: temperature (22°C vs. 28°C): ANOVA, F1,28 = 6.958, P = 0.0135
Ler vs. fil-5: ANOVA, F1,21 = 34, P < 0.001
Ler: Early vs. Late meristems: ANOVA, F1,12 = 89, P < 0.001

Table 4: Floral meristem size

|            | Early meristem mean ± SE (μm) | Late meristem mean ± SE (μm) |
|------------|-------------------------------|-------------------------------|
| Col-7      | n = 5 45 ± 0                  | n = 3 42 ± 1.7                |
| fil-9      | n = 4 80 ± 4.1                | n = 7 64 ± 2.5                |

Col-7 vs. fil-9: Two sample T-test, t = 6.906255, DF = 17, P < 0.001
during the first three weeks, strongly indicates that the filaments are not considered by the plants as true flowers.

Discussion

Fil encodes a putative transcription factor that is a member of the YABBY family. YABBY genes play a demonstrated role in regulating abaxial-adaxial polarity in lateral organs [12,38]. From studies of YABBY genes, it is emerging of late that Fil also serves a role in shoot meristem organization [24,29]. In the inflorescence, unlike the vegetative parts of the plant, Fil functions, at least partially, in non-redundant manner, such that mutations in the gene lead to visually perceptible phenotypes. These phenotypes have been reported for several fil alleles in the Ler background [12,25-27]. In our study, we have characterized a new fil allele in the Col background. Through meticulous analysis of reproductive development, we demonstrate a linkage between distortions in fil IM and FM and the inflorescence and floral abnormal phenotypes. We further propose an explanation as to how these are causally related, thus highlighting the potential of using fil mutants to study meristem organization and its effect on flower morphogenesis.

We have shown that the shape and size of both the IM and FM is altered in fil-9 mutant plants (Figure 3), and that this results from an increase in cell number rather than in cell size. These alterations are associated with defects in primordia initiation, such that several floral bud primordia and multiple floral organs can emerge simultaneously. The changes in the FM presumably prevent the establishment of defined whorls and lead to disruption of the orderly allocation of cells to the emerging organs. We propose that as a result of the blurring of the boundaries between different whorls, the fil-9 flowers developed the observed defects in organ identity. The hypothesis that Fil does not function as a regulator of the expression of organ identity genes per se is supported by the fact that we never observed full organ conversion, while alterations in organ identity occurred only occasionally.

The organ growth defects observed in fil mutants are unlikely to be the result of a simple change in meristem size. In mutants affecting meristem size, such as clavata or wuschel, changes in meristem size indeed lead to an alteration in organ number. The shape and identity of the organs remain, however, unaltered [39-41]. Moreover, although fil meristems are larger than are those in wild type plants, the floral defects are mostly displayed in reduced number and size of the floral organs. The variable defects in floral organ formation and organization in fil-9 mutants (Figure 2) resemble those produced by mutations in tousled, a gene that encodes a protein kinase [42,43]. Likewise, the high variability in the number and morphology of fil floral organs (Figure 2) suggests that disruption of the cell proliferation process occurs within localized areas of activity in the meristem.

The defects we observed are consistent with the suggestion that Fil plays a role in regulating the partitioning of the IM and FM into distinct domains. A number of genes have been implicated in establishing meristematic domains by regulating the specification of the CZ, the boundary between proliferating and differentiating cells, or the development of organ primordia at the periphery of the meristem [reviewed in [2]]. In addition, auxin flux across the meristem is critical for normal patterning, with feedback between genes regulating auxin distribution and those regulating meristematic domains taking place. This suggests an intimate connection between these pathways [2,44]. For instance, mutations in boundary genes, such as BOP1 and BOP2, as well as mutations in auxin influx carriers produce inflorescence and floral defects similar to those we observe in fil-9 mutants [45-48]. Based on these similarities in phenotype, in conjunction with the defects observed in IM and FM organization, we suggest that Fil is required for the appropriate delineation of CZ and PZ boundaries within these meristems. The disorderly emergence of organ primordia in fil-9 plants can be explained by disruption of these boundary domains. Many of these organs are, however, filamentous. Note that when these organs were not included when counting the number of flowers produced by fil-9 inflorescences, the number of flowers in the mutant was the same as in wild type plants, throughout most of the reproductive phase (Figure 4). Sawa et al. have previously shown that similar filamen-
tous structures in the fil-1 mutant express the floral meristem identity gene, *API*, but gradually lose the expression of the floral meristem identity gene, *LEAFY* [26]. Together, these results suggest that the filamentous organs produced by fil IM have a floral meristem identity, although this is insufficient to maintain floral identity. The disruption of meristem organization can also explain the occasional development of a cryptic bract - a normally arrested organ - in fil mutants (Figure 2 and [26]).

Verifying the involvement of *FIL* in the establishment and maintenance of IM boundaries awaits analysis of expression patterns of meristem, primordia and boundary markers, such as *STM, ANT, CUC2* and *UFO*, in fil inflorescences. For example, it was shown by Goldschmidt and colleagues (2008) that altering *FIL* and *YAB3* expression leads to changes in the expression of lateral suppressor, a gene which is normally expressed at the boundary of organ primordia [24]. In this regard, it could be informative to compare the expression patterns of meristem boundary markers in fil-9 which is in the Col background versus other reported fil alleles that are in the Ler background. Differences have been noted in the shape of wild type inflorescence meristems in different *Arabidopsis* ecotypes in terms of height and width though not in the internal organization of layers and zones [49]. It therefore, would be of interest to test whether these different genetic backgrounds differentially sensitize meristem-primordia signaling pathways.

The variable homeotic transformations of floral organs suggest that, similar to IM, fil floral whorls boundaries are also likely to be disrupted. It was previously suggested that *fil* might be a direct regulator of floral organ identity genes [25,26]. However, the range of mosaic organs that develop within each flower point towards shifts in the expression domains of the identity genes as a result of misallocation of cells to the different whorls. This could be tested by examining the expression patterns of the floral organ identity genes and whorl boundary genes, such as *SLIP*, in fil flowers. Previous studies on fil alleles have presented analyses of expression of the floral organ identity genes, *API, APETALA3, PISTILLATA* and *AGAMOUS* in fil flowers [25,26]. Nevertheless, such analyses were limited to specific types of fil abnormal flowers and did not address the wide range of floral organ phenotypes that exist in fil mutants. Thus, the complex alteration in expression patterns of the organ identity genes that occurs could not be fully revealed.

In an attempt to explain the formation of mosaic organs in flowers of several *ap2* and *ag* alleles, Bowman et al. suggest that the primordia of these organs encompass more than a single geographic whorl, resulting in organs composed of cell arising from different whorls [50]. We suggest that in fil, whorl boundaries are erratic, resulting in organs with abnormal shapes and mixed identities.

An additional support for the causal relationship between the disrupted meristem organization and the various phenotypes of fil floral organs is that these phenotypes are more severe in later-arising inflorescences and flowers (Tables 1, 2). Such acropetal changes in fil-9 mutant phenotypes are correlated with changes in meristem size and shape. We have documented that meristems at a late developmental stage are smaller than those at an earlier stage, both in wild type and in fil inflorescences (Figure 3, Table 3). It is hence possible that the age-dependent depletion of the meristematic pool intensifies the distortion of the inflorescence and floral meristems in fil-9 plants and thus, their floral phenotypes.

**Conclusions**

Through the characterization of a new fil allele, fil-9, we have re-evaluated the role of *FIL* in the organization of IMs and FMs and in the emergence of reproductive organ primordia. fil-9 floral organs are highly variable in terms of organ number, size and morphology. In addition, *fil*-9 plants show disruption of floral whorl boundaries, with floral organs occasionally displaying mixed identities. The high variability of the floral aberrations indicated that they are the result of underlying defects in the meristem. Indeed, both the inflorescence and floral meristems of *fil*-9 plants are larger (i.e. containing more cells) and are distorted, as compared to these meristems in wild type plants, and produce several primordia in a simultaneous rather than a sequential manner. Moreover, the age-dependent decrease in meristem size was correlated with enhanced severity of floral phenotypes. Overall, our results support the role of *FIL* in the organization of the meristem and provide new insight into the relationship between meristem organization and floral form.

**Methods**

**Plant materials and growth conditions**

The *fil*-9 line was isolated from activation-tagged lines [31] in a Col-7 background. These seeds, as well as wild type Col-7 seeds, were obtained from The Nottingham *Arabidopsis* Stock Center (University of Nottingham, UK). *fil*-5 and *fil*-8 seeds were a kind gift from Yuval Eshed, Weizmann Institute of Science, Rehovot, Israel. *pPIN1::PIN1-GFP* seeds were plated on a mixture of soil (sphagnum peat and tuff) and perlite (2:1) and grown under either a 16 h light/8 h dark cycle or constant light (*pPIN1::PIN1-GFP*) at 22°C or 28°C, as indicated.

For the characterization of the *fil*-9 phenotype, a total of 70 early-arising (flowers 1-10) and 162 late-arising (flower 11 and later) flowers from 7 plants grown at 22°C were analyzed. A total of 172 flowers from 3 *fil*-9 plants grown at 28°C were analyzed for the temperature-dependency comparison. To measure the number of flowers produced on *fil*-9 plants, as compared to the wild type,
the main inflorescences of 17 \textit{fil}-9 and 20 wild type plants were analyzed during 6 weeks from bolting.

**Light, scanning and confocal electron microscopy**
Fresh tissue was dissected and examined with a SMZ800 dissecting microscope (Nikon) and pictures were taken with an attached Coolpix 4500 digital camera (Nikon).

For scanning electron microscope (SEM), flowers were fixed in 3.7% formaldehyde, 50% ethanol, and 5% acetic acid for 8 h and dehydrated in a graded ethanol series. Dehydrated flowers were critical point dried in liquid CO$_2$ and sputter-coated with gold palladium. Specimens were analyzed and photographed with a JSM5610LV scanning electron microscope (Jeol, Japan).

Fresh meristems were dissected into GM medium and incubated with FM4-64 (Invitrogen™) at a final concentration of 10 μM for 10-20 min. All imaging was done using a Zeiss LSM 510 Meta confocal microscopy system using a waterdipping 40× objective. GFP was excited with a 488 nm Argon laser, with emission detection through the meta-channel at 497 to 550 nm.

**Measurements of meristem size and cell number**
Meristem size measurements were performed from the SEM scans. The FM measurements were performed in developing flowers, containing only sepal primordia, with mesritem size being determined as the length between opposite sepals.

Cell number was counted in a circle of 966 μm$^2$, an area that covered most of the wild type inflorescence meristem. We used the \textit{pPIN1::PIN1-GFP} construct [36] (in the background of the wild type or \textit{fil}-9 plants) as a marker for the meristematic L1 cell layer.

For the statistical analysis, we employed two-way Analysis of Variance (ANOVA) or two sample T-test, as indicated. Analyses were done using SYSTAT v. 11 (SYSTAT Software, San Jose, CA).

**DNA and RNA extraction and RT-PCR**
Plant DNA was extracted using the CTAB extraction method [51]. Mapping of the T-DNA insertion in \textit{fil}-9 plants was performed by PCR amplification using a T-DNA specific primer (tsp1 5′-ACG ACG GAT CGT AAT GTA CAG CAA CCA CAT C-3′) and a DNA specific primer (infilrv 5′-TCT CTC TG-3′), and reverse primers FILP2 5′-AGA TTG GTA CAG CAA CCA CAT C-3′ or FILP4 5′-CCA ACG TTA GCA GCT GCA GGA-3′, and \textit{Tubulin4} (At5g44340) primers (TUB4F 5′-CCG TGC AAT ACG TCG TGG-3′ and TUB4R 5′-TCA GAG ACC TTA GGA GAA GG-3′) as control.

**Authors’ contributions**
NL conducted the genetic molecular analyses to map the mutation and all the scanning electron and confocal microscopy studies. NN participated in the isolation of the mutant, analyzing and photographing the specimens, and writing the manuscript, RB did the mutant floral phenotyping, MZ designed and supervised the study and wrote the manuscript. All authors read and approved the final manuscript.

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