



HAL
open science

SUPERMAN prevents class B gene expression and promotes stem cell termination in the fourth whorl of *Arabidopsis thaliana* flowers

Nathanaël Prunet, Weibing Yang, Pradeep Das, Elliot M. Meyerowitz,
Thomas P. Jack

► **To cite this version:**

Nathanaël Prunet, Weibing Yang, Pradeep Das, Elliot M. Meyerowitz, Thomas P. Jack. SUPERMAN-prevents class B gene expression and promotes stem cell termination in the fourth whorl of *Arabidopsis thaliana* flowers. *Proceedings of the National Academy of Sciences of the United States of America*, 2017, 114 (27), pp.7166-7171. 10.1073/pnas.1705977114 . hal-01592225

HAL Id: hal-01592225

<https://hal.science/hal-01592225v1>

Submitted on 26 May 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Copyright



SUPERMAN prevents class B gene expression and promotes stem cell termination in the fourth whorl of *Arabidopsis thaliana* flowers

Nathanaël Prunet^{a,b,1}, Weibing Yang^c, Pradeep Das^d, Elliot M. Meyerowitz^{b,1}, and Thomas P. Jack^a

^aDepartment of Biological Sciences, Dartmouth College, Hanover, NH 03755; ^bDivision of Biology and Biological Engineering, Howard Hughes Medical Institute and California Institute of Technology, Pasadena, CA 91125; ^cSainsbury Laboratory, University of Cambridge, Cambridge CB2 1LR, United Kingdom; and ^dLaboratoire de Reproduction et Développement des Plantes, Université Lyon 1, Centre National de la Recherche Scientifique, Institut National de la Recherche Agronomique, École Normale Supérieure de Lyon, 69364 Lyon cedex 07, France

Contributed by Elliot M. Meyerowitz, May 19, 2017 (sent for review April 11, 2017; reviewed by Richard G. H. Immink and David Smyth)

The molecular and genetic networks underlying the determination of floral organ identity are well studied, but much less is known about how the flower is partitioned into four developmentally distinct whorls. The *SUPERMAN* gene is required for proper specification of the boundary between stamens in whorl 3 and carpels in whorl 4, as *superman* mutants exhibit supernumerary stamens but usually lack carpels. However, it has remained unclear whether extra stamens in *superman* mutants originate from an organ identity change in whorl 4 or the overproliferation of whorl 3. Using live confocal imaging, we show that the extra stamens in *superman* mutants arise from cells in whorl 4, which change their fate from female to male, while floral stem cells proliferate longer, allowing for the production of additional stamens.

flower development | boundary formation | floral organ identity | stem cells | *SUPERMAN*

Most of the body of flowering plants is generated post-embryonically from apical meristems, which are pools of undifferentiated cells at the tips of stems and roots. Divisions in the shoot apical meristem (SAM) allow for the continuous production of lateral organs on the flanks of the stem: leaves during the vegetative phase, then floral meristems (FMs) after the SAM transitions to the reproductive phase. FMs, in turn, generate the floral organs that comprise the flower.

Partition of the organism into distinct tissues and organs is a fundamental process of development in both animals and plants, yet it relies on different mechanisms in each kingdom. In animals, tissue separation is determined by cell surface cues that influence the adhesive properties of cells and their ability to interact with each other (1). Unlike animal cells, however, plant cells are surrounded and connected to their neighbors by contiguous cell walls that prevent them from migrating. As new organs form, they are separated from surrounding tissues by a boundary, which consists of a group of cells with restricted growth that act as a physical barrier separating two different developmental programs (2).

In *Arabidopsis thaliana*, whereas the SAM gives rise to lateral organs one at a time, in an iterative, spiral pattern, the FM semi-synchronously produces 16 floral organs, with four different identities, in four adjacent whorls. Floral organ identity is determined by the combinatorial action of four classes of MADS-box transcription factors, which form distinct complexes in the four floral whorls (3, 4). For instance, a combination of APETALA3 and PISTILLATA (AP3 and PI, class B), together with AGAMOUS (AG, class C) and SEPALLATA3 (SEP3, class E) specifies stamens in whorl 3, whereas complexes composed solely of AG and SEP3 trigger carpel development in whorl 4. Targets of these MADS-box transcription factors have been extensively studied, and downstream regulatory networks have been partially deciphered (4). However, the mechanisms that underlie the patterning of the FM, with the generation of four distinct types of organs in such a

constrained space and time, remain poorly understood. In particular, how boundaries between the floral whorls are established is still unclear. Here, we analyze the role of *SUPERMAN* (*SUP*) in defining the boundary between whorl 3 stamens and whorl 4 carpels.

SUP encodes a transcriptional repressor with a C2H2 zinc-finger DNA-binding domain and an EAR repression domain (5–8), and is expressed at the boundary between whorls 3 and 4 (6, 9). *sup* mutant flowers have numerous extra stamens, whereas carpel tissue is usually reduced or missing (10, 11). This phenotype is associated with the expansion of AP3 and PI expression closer to the center of the FM compared with the wild type (10). Overall, floral organ number is higher in *sup* flowers than in the wild type, indicating an increase in cell proliferation in developing *sup* flower buds. Although *SUP* was first characterized a quarter century ago, there are still two conflicting models to explain *SUP* function and the developmental origin of the *sup* phenotype. Here, we refer to these two models as “whorl 3” and “whorl 4” models, based on the whorl where the extra stamens in *sup* mutant flowers hypothetically form. The whorl 4 model proposes that *SUP* functions to prevent ectopic expression of AP3 and PI in whorl 4. According to this model, ectopic AP3/PI expression in whorl 4 of developing *sup* flowers triggers the formation of stamens instead of carpels, and prolongs cell proliferation in the FM (10, 11). Conversely, the whorl 3 model proposes that *SUP* controls the balance of cell proliferation between whorl

Significance

The *Arabidopsis thaliana* flower is a complex structure that consists of discrete floral organs (sepals, petals, stamens, and carpels) that are separated by regions lacking organ growth called boundaries. The *SUPERMAN* (*SUP*) gene functions to define the boundary between the male organs (stamens) and female organs (carpels) in the flower. Previous work on boundary formation in plants has focused on growth repression, rather than on identity separation. Using live confocal imaging, we demonstrate that *SUP* functions by keeping the male and female developmental programs spatially and temporally separate, which is critical for the fertility of the flower. In addition, we show a second role of *SUP* in the timely termination of floral stem cells.

Author contributions: N.P., P.D., E.M.M., and T.P.J. designed the experiments; N.P., W.Y., and T.P.J. performed the experiments; N.P. and T.P.J. generated the reporter lines; N.P. performed the live confocal imaging; W.Y. performed the in situ hybridizations; N.P. wrote the paper; and P.D., E.M.M., and T.P.J. edited versions of the paper.

Reviewers: R.G.H.I., Wageningen Plant Research; and D.S., Monash University.

The authors declare no conflict of interest.

¹To whom correspondence may be addressed. Email: nprunet@caltech.edu or meyerow@caltech.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1705977114/-DCSupplemental.

3 and 4, and suggests that production of extra stamens in *sup* mutant flowers results from increased cell proliferation in whorl 3 at the expense of whorl 4 (6, 12). In this study, we used live confocal imaging to investigate the developmental basis of the *sup* phenotype. We show that extra stamens in *sup* mutant flowers arise from a subset of whorl 4 cells that switch identity from female to male, as predicted by the whorl 4 model, and that the floral stem cells at the center of the flower, rather than cells in whorl 3, are the source of the overproliferation observed in *sup* mutants.

Results

SUP Is Expressed on Both Sides of the Boundary Between Whorls 3 and 4. We generated a *gSUP-3xVenusN7* translational *SUP* reporter that complements the *sup-1* mutant phenotype. The *SUP* protein is first detected at stage 3, in cells adjacent to the boundary between whorls 3 and 4, inside of lateral sepal primordia (Fig. 1 *A* and *B*; stages as described in ref. 13), and quickly expands to form

an oblong ring ~3 to 4 cells wide, and longer medially than laterally (Fig. 1 *A* and *C*). At early stage 5, *SUP* is detected on both sides of the boundary between whorls 3 and 4, which at this stage forms a groove between the developing stamen primordia and the center of the flower (Fig. 1 *D*). By late stage 5, *SUP* expression becomes restricted to a narrower band of cells at the boundary (Fig. 1 *A*). *gSUP-3xVenusN7* fluorescence appears to peak at stage 4, before decreasing in intensity during stage 5 and becoming undetectable by late stage 6 (Fig. 1 *A*). Overall, the *SUP* expression pattern resembles that of *AP3*, but *SUP* appears to accumulate closer to the center of the flower (Fig. 1, compare *A* and *E*). To determine more precisely where *SUP* is expressed relative to the boundary between whorls 3 and 4, we monitored the expression of *SUP* and class B genes simultaneously, using the *gSUP-3xVenusN7* reporter together with a *gAP3-GFP* translational reporter (Fig. 1 *F*), a *gPI-GFP* (Fig. S1) translational reporter, or a *pAP3-CFPN7* transcriptional reporter (Fig. 2). *SUP* expression initiates

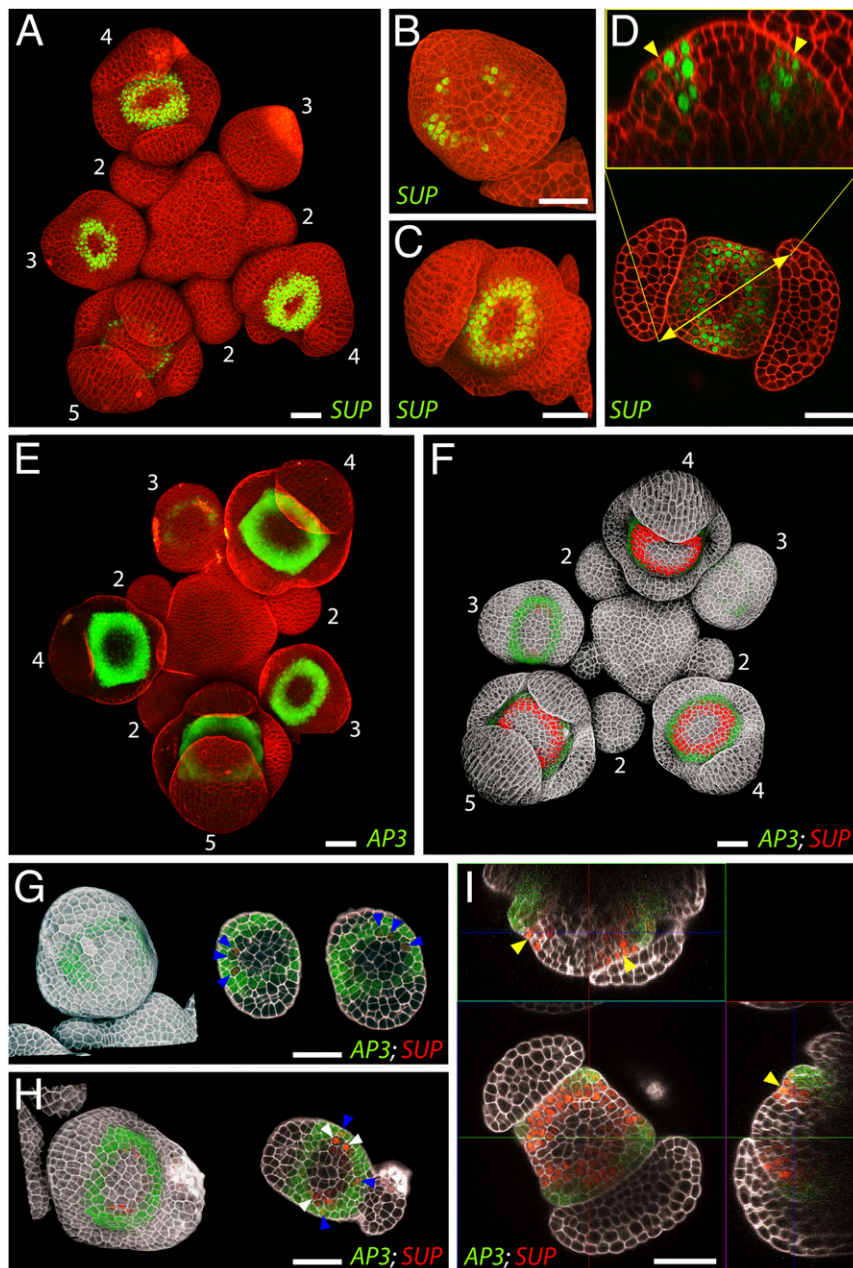


Fig. 1. Expression of *SUP* and *AP3* in wild-type flowers. Expression of the *gSUP-3xVenusN7* (*A–D*) and *gAP3-GFP* (*E*) reporters separately (*A–E*), or together (*F–I*). (*A*, *E*, and *F*) Whole inflorescences; numbers indicate floral stages. (*B–D* and *G–I*) Flower buds at early stage 3 (*B* and *G*), late stage 3 (*H*), stage 4 (*C*), and stage 5 (*D* and *I*). *A*, *E*, *F*, and *G* and *H*, *Left* show maximum intensity projections (MaxIPs). *D*, *Lower*; *G* and *H*, *Right*; and *I*, *Lower Left* show slice views along horizontal planes. *D*, *Upper* and *I*, *Upper Left* and *Lower Right* show slice views along vertical planes. Yellow arrowheads indicate the position of the boundary between whorls 3 and 4, blue arrowheads mark cells that express both *AP3* and *SUP*, and white arrowheads mark cells that express *SUP* but not *AP3*. (Scale bars: 25 μ m.)

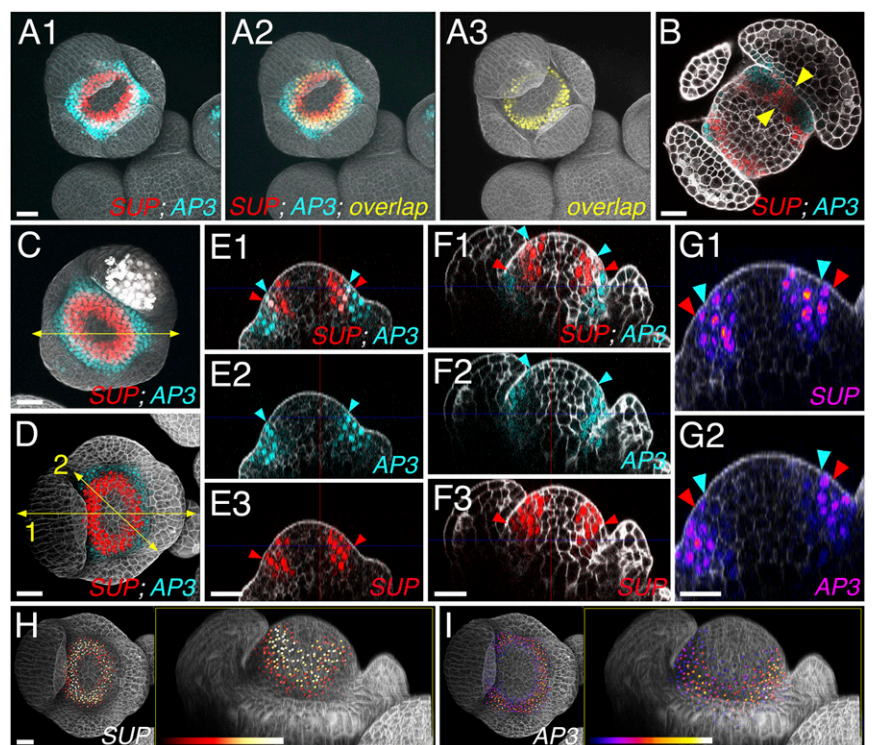
shortly after that of AP3 at stage 3 (Fig. 1F), and the first cells to express SUP also express AP3 (Fig. 1G), indicating that SUP is initially expressed in whorl 3. However, from late stage 3 on, we observed cells that express SUP but do not express AP3 or PI (Figs. 1H and I and 2E1–F3 and Fig. S1), demonstrating that SUP expression expands into whorl 4. At stages 4 and 5, SUP is clearly found on both sides of the boundary between whorls 3 and 4 (Figs. 1I and 2A1–F3 and Fig. S1B–D). SUP accumulation overlaps with that of AP3/PI in whorl 3 at the boundaries between stamen primordia (Fig. 2B, D, and F1–F3, Fig. S1D, and Movie S1), and in a narrow, one- to two-cell-wide band on the adaxial side of stamen primordia (Figs. 1I and 2C and E1–E3, Fig. S1D, and Movie S1). SUP is also expressed without AP3/PI in another narrow, one- to two-cell-wide band in the outer part of whorl 4 (Figs. 1I and 2A2 and E1–E3, Fig. S1D, and Movie S1). Together, these data clearly show that, contrary to earlier interpretations (6), the SUP protein accumulates on both sides of the boundary between whorl 3 stamens and whorl 4 carpels, and is not confined solely to whorl 3. Double fluorescence in situ hybridization experiments for SUP and AP3 confirmed that this is also the case at the mRNA level (Fig. S2). Indeed, SUP protein levels appear higher in whorl 4, where the AP3 and PI proteins do not accumulate (Fig. 2G1 and H). Similarly, AP3 expression appears stronger in whorl 3 cells that do not express SUP (Fig. 2G2 and I).

To better understand where SUP is expressed relative to the positions where stamen and carpel primordia initiate, we examined plants expressing both the *gSUP-3xVenusN7* reporter and the *DORNROSCHEN-LIKE (DRNL) pDRNL-erGFP* reporter, which marks floral organ founder cells (Fig. S3A and B) (14). In particular, DRNL expression in whorl 3 forms a ring at early stage 4 that is reminiscent of AP3 and SUP expression patterns, before being restricted to foci at the sites of stamen initiation at stage 5 (Fig. S3A). At that stage, DRNL is also expressed in two

foci in whorl 4, which correspond to the sites of carpel initiation, and in two narrow arcs of cells connecting these foci (Fig. S3B and C) (14). SUP and DRNL expression partially overlap in stamen primordia in whorl 3 (Fig. S3A and B), whereas a narrow ring of SUP accumulation in whorl 4 directly surrounds DRNL expression in carpel founder cells in the center of whorl 4 (Fig. S3B).

Extra Stamens in *sup-1* Flowers Arise from Whorl 4 Cells. To determine whether the extra stamens in *sup* mutant flowers arise from whorl 3 or whorl 4, we compared the expression of class B genes in wild-type and *sup-1* flowers by using a *pAP3-3xVenusN7* transcriptional reporter (Fig. 3) and the *gAP3-GFP* (Fig. S4) and *gPI-GFP* translational reporters (Fig. S5). At stages 3 and 4, AP3 expression appears similar in the wild type and in *sup-1* (Fig. 3, compare A and B and Fig. S4, compare A and B). However, by stage 5, both AP3 and PI are expressed closer to the center of the flower in *sup-1* than in the wild type (Fig. 3, compare A and B; and Figs. S4, compare A and B; and S5, compare A and B) (10). Whereas the fourth whorl of wild-type flowers shows no AP3 expression or PI accumulation (Fig. 3C and E and Fig. S5C), a narrow, two-cell-wide band of AP3/PI expression can be seen inside of the boundary between stamen primordia and the center of *sup-1* flowers at stage 5 (Fig. 3D and F and Fig. S5D). At stage 6, the whole fourth whorl of wild-type flowers develops into carpel primordia (Fig. 3C) (13). Conversely, in *sup-1* flowers, extra stamen primordia only start forming within the ring of extra AP3-expressing cells at stage 7, with a slight delay compared with wild-type carpels (Fig. 3G; stages for *sup-1* flowers were determined based on time elapsed after stage 5, which is the last stage at which wild-type and *sup-1* flowers are morphologically identical). As these extra stamens develop, AP3 expression spreads again beyond the boundary of the primordia toward the center of *sup-1* flowers, forming another narrow ring of AP3-expressing cells, which later gives rise to

Fig. 2. Overlap between SUP and AP3 expression patterns. All images show wild-type flowers expressing the *gSUP-3xVenusN7* and *pAP3-CFPN7* reporters; cell walls were stained with propidium iodide (gray); SUP expression is shown in red and AP3 expression in green, except in G1 and G2, where the intensity of the *gSUP-3xVenusN7* (G1) and *pAP3-CFPN7* (G2) signal is indicated by a fire color code: The brighter the color, the stronger the signal; yellow in A2 and A3 marks the overlap between SUP and AP3 expression, as detected with the Imaris software. (A1–A3) MaxIPs of a stage 4 flower, showing the expression of SUP and AP3 alone (A1), together with the overlap between SUP and AP3 expression alone (A2), and the overlap between SUP and AP3 expression alone (A3). (B) Slice view of a stage 5 flower along a horizontal plane; yellow arrowheads mark the boundary between two medial stamens. (C and D) MaxIPs of stage 4 flowers. (E1–E3) Views of a vertical optical section along the yellow arrow in C, showing the expression of SUP and AP3 together (E1) or separately (E2 and E3); this section goes through opposite medial stamen primordia. (F1–F3) Views of a vertical optical section along yellow arrow number 1 in D, showing the expression of SUP and AP3 together (F1) or separately (F2 and F3); this slice goes through the boundaries between medial stamens. (G1 and G2) Views of a vertical optical section along yellow arrow number 2 in D, showing the intensity of the *gSUP-3xVenusN7* (G1) and *gAP3-GFP* (G2) signal. Red arrowheads in E1–G2 mark the outer boundary of the SUP expression domain; cyan arrowheads mark the inner boundary of AP3 expression domain. Thus, the area between the arrows marks the expression overlap of SUP and AP3. (H and I) Apical (Left) and lateral (Right) views of a stage 4 flower bud showing the nuclei expressing SUP (H) and AP3 (I) as detected with Imaris, with expression intensity indicated by color codes. (Scale bars: 20 μ m.)



more stamen primordia (Fig. 3K). This iterative process allows for the formation of several consecutive rings of stamens, sometimes resulting in flowers with more than 20 stamens. It is worth noting that *AP3* is never expressed throughout the center of *sup-1* flowers, which eventually develop into stunted, misshapen carpels or chimeric stamen/carpel organs (10, 11). Accordingly, organ primordia, composed both of cells that express *AP3* and cells that do not, can often be seen in the center of developing *sup-1* flowers (Fig. S6 A and B).

We sought to establish whether the extra *AP3*-expressing cells in stage 5 *sup-1* flowers derive from whorl 4 cells that change identity, or from whorl 3 cells that overproliferate. The ring of extra *AP3*-expressing cells in *sup-1* flowers looks similar to the ring of *SUP*-expressing cells in whorl 4 of wild-type flowers (compare Fig. 3F to Fig. 1 D and I), suggesting that the loss of *SUP* function might cause ectopic expression of *AP3* in these cells. Using time-lapse imaging of *sup-1 pAP3-3xVenusN7* flower buds, we identified numerous individual cells at the boundary between whorls 3 and 4 that do not express *AP3* at stage 4 but begin to express *AP3* de novo at stage 5 (Fig. 3, compare H and I). These cells that switch identity from female-fated, non-*AP3*-expressing cells to male-fated, *AP3*-expressing cells are situated inside of the boundary between whorl 3 stamens and the center of the flower, indicating that they belong to whorl 4 (Fig. 3J). These data clearly show that the extra *AP3*-expressing cells in *sup-1* flowers originate from whorl 4 cells that switch fate from female

to male, rather than from whorl 3 cells that overproliferate, and supports the whorl 4 model.

Stem Cell Termination Is Delayed in *sup-1* Flowers. The respecification of a small ring of cells in the fourth whorl of *sup-1* flowers at stage 5 is not sufficient to explain the formation of so many supernumerary stamens. The iterative production of rings of extra stamens in the fourth whorl of *sup-1* flowers requires an increase or prolongation of cell proliferation compared with the wild type. To test whether cells in the floral meristem are the source of overproliferation in *sup-1* mutants, we monitored the expression of stem cell marker *CLAVATA3* (*CLV3*) and stem cell-promoting gene *WUSCHEL* (*WUS*) by using *pCLV3-erGFP* (15) and *pWUS-erGFP* transcriptional reporters. *CLV3* expression persists in wild-type flowers through stage 6 (Fig. 4A) (16), but is no longer detectable at stage 7, as stem cells are incorporated into developing carpels (Fig. 4B). Conversely, we observed *CLV3* expression as late as stage 10 in a small dome at the center of *sup-1* flowers, after several extra stamens have formed (Fig. 4C). Stem cell termination is thus clearly delayed in *sup-1* flowers compared with the wild type. Similarly, *WUS* expression stops by stage 5 in wild-type flowers (17), but is maintained much longer in some *sup-1* flowers (Fig. 4D), indicating that a bona fide FM remains functional in *sup-1* flowers longer than it does in the wild type. *AG* is responsible for triggering stem cell termination in wild-type flowers by turning off the expression of *WUS* (18–21), and most mutants with a delay or loss

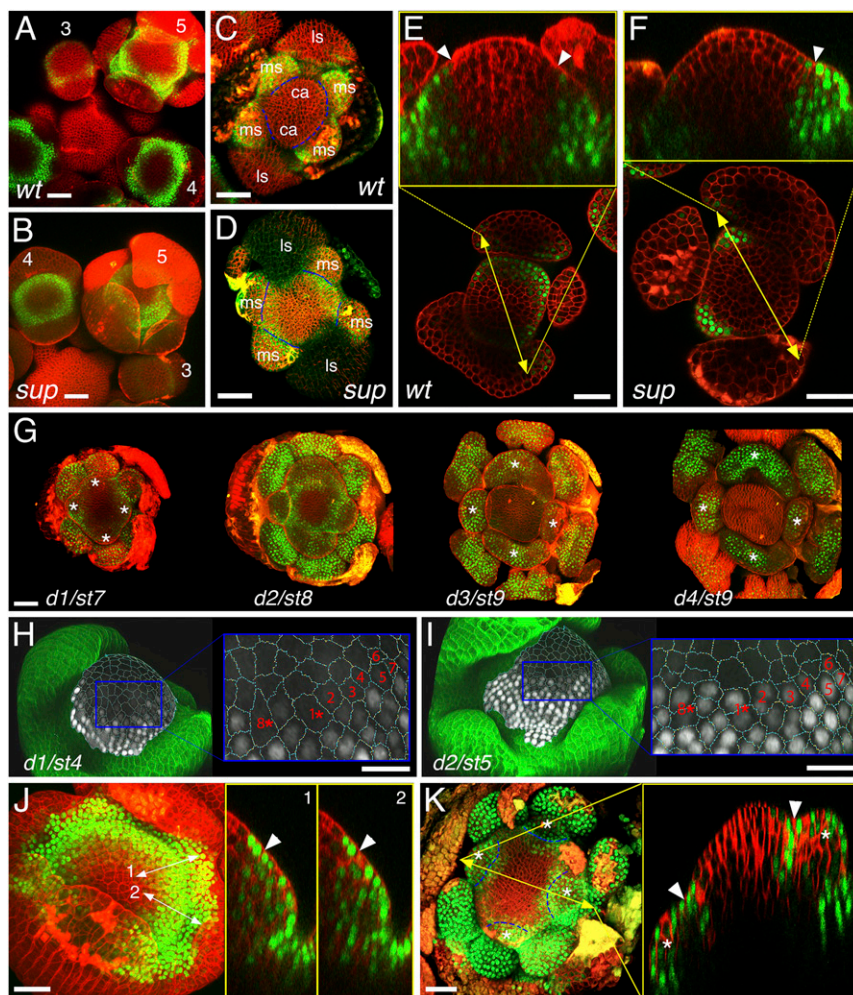


Fig. 3. Expression of *AP3* in wild-type and *sup-1* flowers. Expression of the *pAP3-3xVenusN7* reporter in the wild type (A, C, and E) and *sup-1* (B, D, and F–K). (A and B) Whole inflorescences; numbers indicate floral stages. (C and D) Stage 5 flowers after removal of medial sepals; ca, carpel; ls, lateral sepal, covering lateral stamen; ms, medial stamen; dotted blue lines mark the boundary between whorl 3 stamens and the center of the flower. (E and F) Stage 5 flowers, slice views along horizontal planes (Lower) and vertical planes (Upper); white arrowheads mark the boundary between whorl 3 stamens and the center of the flower. (G) Four-day time lapse of a single *sup-1* flower between stages 7 and 9; white asterisks mark extra stamen primordia. (H and I) Two-day time lapse of an individual *sup-1* flower between stages 4 (H) and 5 (I); Left show a lateral view of the flower, with a segmented projection of the L1 layer in the center; Right show a close-up of the same area on each day; red numbers mark cells that do not express *AP3* at stage 4, but express *AP3* at stage 5; asterisks indicate divisions that occurred between stages 4 and 5. (J) MaxIP (Left) and slice view along vertical planes (Right) of the flower shown in J; white arrowheads mark the boundary between whorl 3 stamens and the center of the flower. (K) MaxIP (Left) and slice view along the vertical planes (Right) of a stage 8 *sup-1* flower; white asterisks mark extra stamen primordia; white arrowheads and dashed blue lines mark the boundary between extra stamen primordia and the center of the flower. (Scale bars: 20 μ m.)

of floral stem cell termination have defects in *AG* expression (4). We thus used the *gAG-GFP* reporter (22) to compare the expression of *AG* in wild-type and *sup-1* flowers, and *AG* expression appears unaffected in *sup-1* flowers (Fig. 4, compare *E* and *F* and *G* and *H*) (10), suggesting that an *AG*-independent mechanism is responsible for the delay in stem cell termination in *sup-1* flowers. *SUP* affects floral stem cells noncell-autonomously, as the *SUP* and *CLV3* expression domains are separated by a narrow, one- to two-cell-wide ring (Fig. 4 *I* and *J*). Indeed, this ring of cells separating the *SUP* and *CLV3* expression domains expresses *DRNL* and likely corresponds to the carpel founder cells (Fig. S3 *D* and *E*). However, the *SUP* expression domain tightly surrounds that of *WUS*, with a few cells expressing both genes, suggesting that the effect of *SUP* on stem cells may be mediated by *WUS* (Fig. 4*K* and Movie S2).

Discussion

It is worth noting that several studies have shown that ectopic expression of *SUP* causes a decrease in cell proliferation (5, 23–25), which was interpreted as evidence in support of the whorl 3 model. It is not surprising, however, for a boundary gene to control cell proliferation, as cell division rates are lower at boundaries, including the boundary between stamens and carpels, than in developing organs (2, 26). For instance, *RABBIT EARS* (*RBE*), which encodes a C2H2 zinc-finger protein related to *SUP*, specifies the boundary between whorls 2 and 3 by excluding *AG* from whorl 2 (27, 28), and also specifies the interspal boundaries by regulating cell proliferation in whorl 1 via the miR164/*CUP-SHAPED COTYLEDON* (*CUC*) module (29).

Similarly, a role for *SUP* in the control of cell proliferation does not exclude the possibility that *SUP* also affects *AP3/PI* expression. Moreover, rates of cell proliferation on either side of the boundary between whorls 3 and 4 appear unaffected in *sup-1* flowers compared with the wild type (26), contrary to the predictions of the whorl 3 model.

Our data confirm, instead, the predictions of the whorl 4 model. Specifically, we show that the extra stamens in *sup* mutant flowers arise from a narrow ring of cells in the outer part of whorl 4, adjacent to the boundary with whorl 3, which change identity from female to male at the transition between stages 4 and 5, and start expressing *AP3* de novo (Fig. 3). Cells in this ring then divide, allowing for the formation of extra stamens. The *sup* phenotype was initially described as heterochronic, *sup* flowers being “stuck in developmental time” (10, 11). The *sup* phenotype is indeed iterative: as extra stamen primordia arise, the lack of functional *SUP* at the inner boundary of these stamens causes *AP3* expression to spread again toward the center of the flower (Fig. 3*K*), allowing for the formation of additional stamens. Even as several rings of extra stamens form one after the other, the center of the flower, which is still devoid of *AP3* expression, is replenished by the floral stem cells, which are maintained longer in *sup* flowers than in the wild type (Fig. 4). Eventually, the center of *sup* flowers differentiates into stunted carpels or mosaic, stamen-carpel organs (Fig. S6). The fact that *SUP* is expressed in the fourth whorl of wild-type flowers, in the same cells that express *AP3* in the fourth whorl of *sup* flowers (Figs. 1 *D* and *I* and 3 *D* and *F*), suggests that *SUP* cell-autonomously represses *AP3* expression in the outer part of whorl 4. Whether

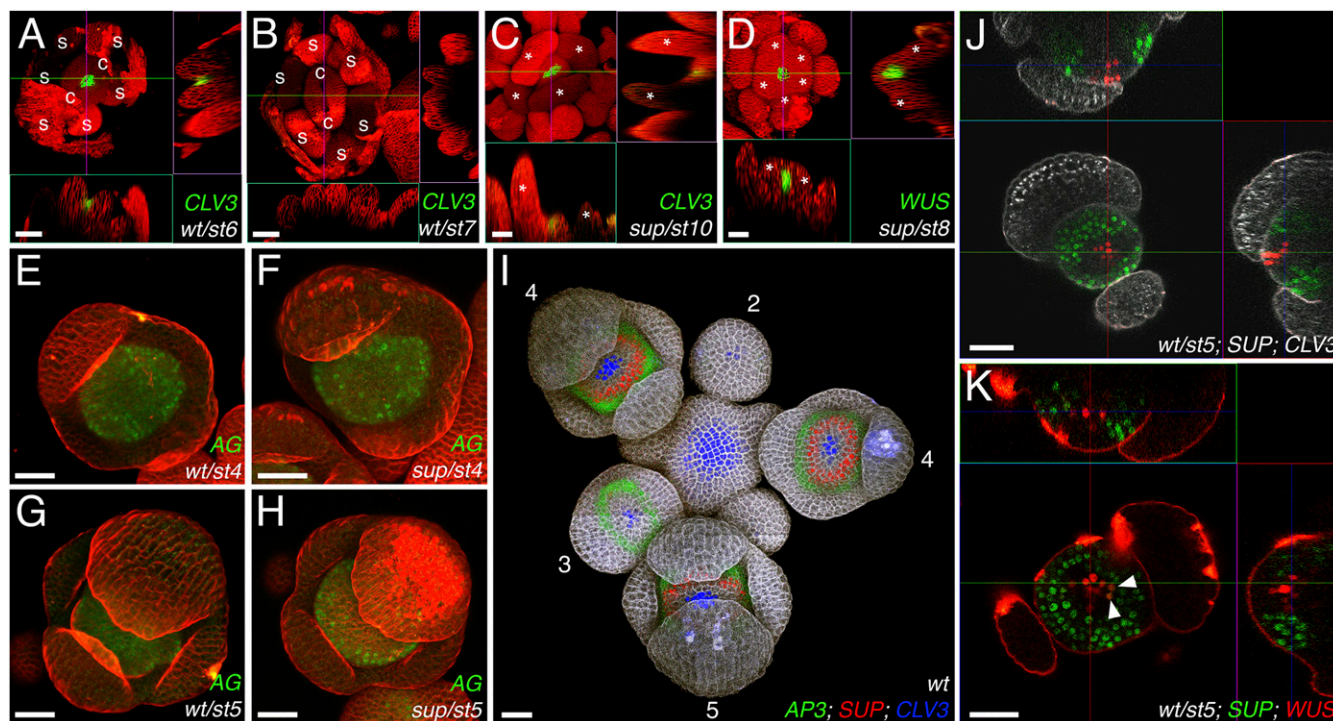


Fig. 4. *SUP* promotes stem cell termination noncell-autonomously, and independently of *AG* expression. (*A–C*) Expression of the *pCLV3-erGFP* reporter in stage 6 (*A*) and 7 (*B*) wild-type flowers, and in a stage 10 *sup-1* flower (*C*); *Upper Left* show MaxIPs, with GFP fluorescence detected with Imaris; *Lower Left* and *Upper Right* show slice views along the *xz* and *yz* planes, respectively; *c*, carpel; *s*, whorl 3 stamen. (*D*) Expression of the *pWUS-erGFP* reporter in a stage 8 *sup-1* flower. Asterisks in *C* and *D* mark extra stamen primordia. (*E–H*) Expression of the *gAG-GFP* reporter in stage 4 (*E* and *F*) and 5 (*G* and *H*) wild-type (*E* and *G*) and *sup-1* (*F* and *H*) flowers. (*I*) Expression of the *gAP3-GFP* (green), *gSUP-3xVenusN7* (red), and *pCLV3-dsRedN7* (blue) reporters in a wild-type inflorescence; numbers indicate floral stages. (*J*) Optical sections of a stage 5 flower expressing the *gSUP-3xVenusN7* (green) and *pCLV3-dsRedN7* (red) reporters; cell walls were stained with propidium iodide (gray); *Lower Left* shows a horizontal section, *Upper Left* and *Lower Right* show vertical sections. (*K*) Optical sections of an early stage 5 flower expressing the *gSUP-3xVenusN7* (green) and *pWUS-dsRedN7* (red) reporters; *Lower Left* shows a horizontal section, *Upper Left* and *Lower Right* show vertical sections; white arrowheads indicate nuclei that express both reporters. (Scale bars: 20 μ m.)

such a repression is direct or indirect, however, remains unknown. Conversely, *SUP* affects floral stem cells noncell-autonomously [the fully complementing *gSUP-3xVenusN7* construct encodes a protein that exceeds the size exclusion limit for passage through plasmodesmata (30), showing that the SUP protein does not need to migrate from cell to cell to accomplish its function], and independently of *AG* expression (Fig. 4). *KNUCKLES (KNU)*, which encodes a C2H2 zinc-finger protein closely related to *SUP*, also promotes the termination of floral stem cells by repressing *WUS* (21). However, while the expression of *SUP* and *WUS* shows a long, but only minor spatial overlap, the expression of *KNU* and *WUS* shows a full, but very transient spatial overlap, as the onset of *KNU* expression at stage 6 directly correlates with the arrest of *WUS* expression (21). *KNU* likely represses *WUS* expression directly, and it is possible that *SUP* also represses *WUS* directly, but this could be the case only at the periphery of *WUS* expression domain. Overall, the effect of *SUP* on *WUS* is largely noncell-autonomous, suggesting that *SUP* does not regulate *WUS* expression directly.

Over the last two decades, considerable progress has been made on the understanding of the mechanisms that underlie the formation of boundaries between different organs and between organs and the meristem, both in the SAM and the FM (2, 4). Numerous genes have been characterized, with some, like the *CUC* genes, involved in the formation of all boundaries, and some,

like *SUP* or *RBE*, involved in the formation of specific boundaries in the flower. However, most of these genes are associated with growth suppression, and not, like *SUP*, with the separation of different identities on either side of the boundary (2, 4). This study provides insights into how a boundary gene partitions two different developmental programs in adjacent organs.

Methods

Inflorescences were prepared for imaging as described in refs. 31 and 32. Fluorescence was monitored using LSM-780 (Carl Zeiss) and A1RSi (Nikon) confocal microscopes, and images were processed with the Zen (Zeiss), NIS-elements (Nikon), Fiji, Imares (Bitplane), and MorphoGraphX software. Pictures of whole inflorescences and stage 9 flower buds, which were too large to image in a single objective field, were composed by combining overlapping Z-stacks of the same specimen. Figures were composed with Adobe Photoshop CS6. Detailed information on plant material, construction of reporter lines, and in situ hybridization is provided in *SI Methods*.

ACKNOWLEDGMENTS. We thank John Chandler and Paul Tarr for providing reporter lines, Toshiro Ito and Frank Wellmer for critical reading of the manuscript, and Ann Lavanway for her tremendous help with live confocal imaging. Funding in the T.P.J. Laboratory was provided by National Science Foundation Grant IOS-0926347; and funding in the E.M.M. Laboratory was provided by the Howard Hughes Medical Institute, the National Institutes of Health Grant R01 GM104244, and the Gordon and Betty Moore Foundation through Grant GBMF3406.

- Fagotto F (2014) The cellular basis of tissue separation. *Development* 141:3303–3318.
- Aida M, Tasaka M (2006) Morphogenesis and patterning at the organ boundaries in the higher plant shoot apex. *Plant Mol Biol* 60:915–928.
- Krizek BA, Fletcher JC (2005) Molecular mechanisms of flower development: An armchair guide. *Nat Rev Genet* 6:688–698.
- Prunet N, Jack TP (2014) Flower development in Arabidopsis: There is more to it than learning your ABCs. *Methods Mol Biol* 1110:3–33.
- Hiratsu K, Ohta M, Matsui K, Ohme-Takagi M (2002) The SUPERMAN protein is an active repressor whose carboxy-terminal repression domain is required for the development of normal flowers. *FEBS Lett* 514:351–354.
- Sakai H, Medrano LJ, Meyerowitz EM (1995) Role of SUPERMAN in maintaining Arabidopsis floral whorl boundaries. *Nature* 378:199–203.
- Dathan N, et al. (2002) The Arabidopsis SUPERMAN protein is able to specifically bind DNA through its single Cys2-His2 zinc finger motif. *Nucleic Acids Res* 30:4945–4951.
- Hiratsu K, Mitsuda N, Matsui K, Ohme-Takagi M (2004) Identification of the minimal repression domain of SUPERMAN shows that the DLELRL hexapeptide is both necessary and sufficient for repression of transcription in Arabidopsis. *Biochem Biophys Res Commun* 321:172–178.
- Ito T, Sakai H, Meyerowitz EM (2003) Whorl-specific expression of the SUPERMAN gene of Arabidopsis is mediated by cis elements in the transcribed region. *Curr Biol* 13:1524–1530.
- Bowman JL, et al. (1992) SUPERMAN, a regulator of floral homeotic genes in Arabidopsis. *Development* 114:599–615.
- Schultz EA, Pickett FB, Haughn GW (1991) The FLO10 gene product regulates the expression domain of homeotic genes AP3 and PI in Arabidopsis flowers. *Plant Cell* 3:1221–1237.
- Sakai H, Krizek BA, Jacobsen SE, Meyerowitz EM (2000) Regulation of SUP expression identifies multiple regulators involved in Arabidopsis floral meristem development. *Plant Cell* 12:1607–1618.
- Smyth DR, Bowman JL, Meyerowitz EM (1990) Early flower development in Arabidopsis. *Plant Cell* 2:755–767.
- Chandler JW, Jacobs B, Cole M, Comelli P, Werr W (2011) DORNROSCHE-LIKE expression marks Arabidopsis floral organ founder cells and precedes auxin response maxima. *Plant Mol Biol* 76:171–185.
- Reddy GV, Meyerowitz EM (2005) Stem-cell homeostasis and growth dynamics can be uncoupled in the Arabidopsis shoot apex. *Science* 310:663–667.
- Fletcher JC, Brand U, Running MP, Simon R, Meyerowitz EM (1999) Signaling of cell fate decisions by CLAVATA3 in Arabidopsis shoot meristems. *Science* 283:1911–1914.
- Mayer KF, et al. (1998) Role of WUSCHEL in regulating stem cell fate in the Arabidopsis shoot meristem. *Cell* 95:805–815.
- Lenhard M, Bohnert A, Jürgens G, Laux T (2001) Termination of stem cell maintenance in Arabidopsis floral meristems by interactions between WUSCHEL and AGAMOUS. *Cell* 105:805–814.
- Lohmann JU, et al. (2001) A molecular link between stem cell regulation and floral patterning in Arabidopsis. *Cell* 105:793–803.
- Liu X, et al. (2011) AGAMOUS terminates floral stem cell maintenance in Arabidopsis by directly repressing WUSCHEL through recruitment of Polycomb Group proteins. *Plant Cell* 23:3654–3670.
- Sun B, Xu Y, Ng KH, Ito T (2009) A timing mechanism for stem cell maintenance and differentiation in the Arabidopsis floral meristem. *Genes Dev* 23:1791–1804.
- Urbanus SL, et al. (2009) In planta localisation patterns of MADS domain proteins during floral development in Arabidopsis thaliana. *BMC Plant Biol* 9:5.
- Bereterbide A, Hernould M, Castera S, Mouras A (2001) Inhibition of cell proliferation, cell expansion and differentiation by the Arabidopsis SUPERMAN gene in transgenic tobacco plants. *Planta* 214:22–29.
- Nandi AK, Kushalappa K, Prasad K, Vijayraghavan U (2000) A conserved function for Arabidopsis SUPERMAN in regulating floral-whorl cell proliferation in rice, a monocotyledonous plant. *Curr Biol* 10:215–218.
- Yun JY, Weigel D, Lee I (2002) Ectopic expression of SUPERMAN suppresses development of petals and stamens. *Plant Cell Physiol* 43:52–57.
- Breuil-Broyer S, et al. (2004) High-resolution boundary analysis during Arabidopsis thaliana flower development. *Plant J* 38:182–192.
- Krizek BA, Lewis MW, Fletcher JC (2006) RABBIT EARS is a second-whorl repressor of AGAMOUS that maintains spatial boundaries in Arabidopsis flowers. *Plant J* 45:369–383.
- Takeda S, Matsumoto N, Okada K (2004) RABBIT EARS, encoding a SUPERMAN-like zinc finger protein, regulates petal development in Arabidopsis thaliana. *Development* 131:425–434.
- Huang T, López-Giráldez F, Townsend JP, Irish VF (2012) RBE controls microRNA164 expression to effect floral organogenesis. *Development* 139:2161–2169.
- Kim I, Kobayashi K, Cho E, Zambryski PC (2005) Subdomains for transport via plasmodesmata corresponding to the apical-basal axis are established during Arabidopsis embryogenesis. *Proc Natl Acad Sci USA* 102:11945–11950.
- Prunet N, Jack TP, Meyerowitz EM (2016) Live confocal imaging of Arabidopsis flower buds. *Dev Biol* 419:114–120.
- Prunet N (2017) Live confocal imaging of developing Arabidopsis flowers. *J Vis Exp* 122:e55156.
- Yang W, et al. (2016) Regulation of meristem morphogenesis by cell wall synthases in Arabidopsis. *Curr Biol* 26:1404–1415.