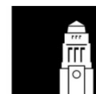
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Walker, CH and Bennett, T orcid.org/0000-0003-1612-4019 (2019) A distributive '50% rule' determines floral initiation rates in the Brassicaceae. Nature Plants, 5. pp. 940-943. ISSN 2055-026X

https://doi.org/10.1038/s41477-019-0503-z

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A distributive '50% rule' determines floral initiation rates in the Brassicaceae

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The spatio-temporal production of flowers is key to determining reproductive fitness in most flowering plants, and yield in many crop species, but the mechanisms regulating this 'reproductive architecture' are poorly characterised. Here we show that in members of the Brassicaceae, total flower number is largely independent of inflorescence number, and the proportion of flowers initiated on the secondary inflorescences represents ~50% of total floral production, irrespective of secondary inflorescence number. This '50% rule' acts as a coordinating principle for reproductive development in Brassicaceae, and similar principles may operate in other species. Our findings suggest that inflorescences continue to compete with each other for a fixed pool of meristematic potential after their activation.

Reproduction in flowering plants consists of a number of hierarchical and sequential developmental phases. Plants must first initiate reproductive branches (inflorescences), and then produce flowers, which upon pollination will give rise to fruit and ultimately seed. To produce an optimal seed set, plants must carefully control the initiation of these organ types in space and time, such that sufficient but not excessive resources are committed to each stage. In the model plant Arabidopsis thaliana (Arabidopsis), the control of inflorescence number is relatively well understood, and exemplifies the classic 'apical dominance' paradigm for the regulation of shoot branching[1]. The

number of inflorescences that Arabidopsis initiates is controlled by the environmental conditions in which the plant is growing; greater resource availability (including light and mineral nutrients) allows for increased initiation of inflorescences[2]. The exact spatio-temporal pattern of inflorescence initiation reflects both developmental history, and the inhibitory 'dominance' effect that actively-growing inflorescences exert over the activation of new inflorescences[2]. However, the principles that govern the number and pattern of flower initiation in Arabidopsis are essentially unknown, and we sought to understand this process.

As a null model, we hypothesised that flower number in Arabidopsis is solely determined by inflorescence number. We examined wild-type Arabidopsis (Col-0) and assessed the number of inflorescences and flowers produced in 15 separate experiments. Comparison of experimental means showed a good correlation between the total inflorescences and total flowers (Fig. 1A), suggesting that a plant producing more inflorescences is capable of supporting a greater number of flowers. However, this relationship only accounted for around 54% of the variation observed ($R^2 = 0.544$), indicating that inflorescence number is not the sole factor regulating flower number. We also examined the relationship between the total flower number and the mean number of flowers per inflorescence (as a proxy for inflorescence meristem activity), and observed no relationship between the two variables ($R^2 = 0.001$) (Fig. 1B). Thus, plants do not regulate flower number solely by altering individual inflorescence meristem activity. These results suggested that total flower number must arise from a more complex combination of both inflorescence number and inflorescence meristem activity. In attempting to understand this, we found a very strong correlation between the number of flowers produced on the secondary inflorescences (see Fig. 1A for definition) and total flower number ($R^2 = 0.930$) (Fig. 1C). Plants typically produced ~50% of their total flowers on the secondary inflorescences, distributing the remaining 50% across the primary inflorescence and higher order inflorescences, regardless of total inflorescence number (Fig. S1, Table S1). We found that this 50% distribution occurs not only in the Col-0 wild type, but also in

both the Ler and Ws-2 ecotypes (Fig. 1D). Furthermore, we found that the 50% distribution was also maintained in the high-branching mutant branched1-2 (brc1-2), and various strigolactone, cytokinin and gibberellin mutants, despite the severe alterations in inflorescence architecture in most of these lines (Fig. 1D).

To assess whether this 50% distribution is robust against physical as well genetic perturbations. We removed either the upper 50%, lower 50% or lower 75% of secondary inflorescences from Col-0 plants during flowering, and allowed the plants to recover. Treated plants initiated new secondary inflorescences, and despite the highly-disruptive perturbations, the secondary inflorescence flower number still tended towards 50% of the total in all treatments, and this proportion was not significantly different from untreated plants in either of the 50% removal treatments (Table S2). This indicates that the mechanism is actively homeostatic during the lifetime of inflorescences, and can correct for perturbations, at least within a certain tolerance range.

Collectively, these data strongly suggested that total flower number is controlled independently of inflorescence number in Arabidopsis. To confirm this, we compared flower production in two high branching mutants, brc1-2 and dwarf14-1 (d14-1) relative to Col-0 wild-type. Despite both mutants producing significantly higher numbers of inflorescences than wild type (Fig. 1E), all three genotypes produced the same number of flowers (Fig. 1F). Taken together, these data suggest that total floral potential is determined independently of inflorescence number, and that each class of inflorescence shares a proportion of the total potential, with secondary inflorescences receiving around 50%. We therefore propose that floral initiation rates between Arabidopsis inflorescences self-organize; the secondary inflorescences continue to mutually inhibit each other following activation, and the more inflorescences there are, the more the activity of each inflorescence meristem is inhibited.

We questioned whether this '50% rule' was a quirk of Arabidopsis reproduction, or was more generalizable, and therefore examined a range of other Brassicaceae species. We examined Brassica napus, a rapid cycling ecotype of Brassica rapa, Cardamine hirsuta (Cardamine), Capsella grandiflora and Capsella rubella. Like Arabidopsis, all these species produce a vegetative rosette, from which a branching system of indeterminate, racemic inflorescences then grows after the floral transition. Despite the qualitative similarities, there are strong quantitative differences in the inflorescence systems between these species, summarised in Fig. S1. We assessed the inflorescence and flower numbers of these species to determine if they follow the same floral distribution as seen in Arabidopsis. Total inflorescence number had no correlation with total flower number across the species ($R^2 = 0.0421$) (Fig. 2A), and similarly, the number of flowers per inflorescence is not correlated with total flower number ($R^2 = 0.032$) (Fig. 2B). The lack of correlation between these parameters is unsurprising given the variation in reproductive architecture between the species. However, when we compared secondary inflorescence flower number and total flower number between these species, there was a clear correlation across species ($R^2 = 0.948$) with ~50% of flowers formed on the secondary inflorescences, irrespective of the underlying architecture (Fig. 2C). This cross-species trend is made even clearer when examining each plant individually ($R^2 = 0.897$) (Fig. 2D).

Thus, as in Arabidopsis, the secondary inflorescences of all examined Brassicaceae spp. typically produced ~50% of the total flowers of the plant. Considering the large differences in reproductive architecture between the species (Supplementary Fig. 1), this is strongly suggestive of a conserved regulatory mechanism in the Brassicaceae acting to distribute inflorescence meristem activity evenly between inflorescences of the same order, regardless how many inflorescences have been produced. Such homeostatic floral distribution cannot be universal in flowering plants, as most species either have determinate inflorescences that produce a small number of flowers, or have unbranched inflorescence systems. However, the underlying mechanism might nevertheless be

conserved across flowering plants. We therefore additionally examined flower number in Myosotis arvensis (forget-me-not; Boraginaceae), a distantly related species with a branching, indeterminate inflorescence system. We found the same strong correlation ($R^2 = 0.932$) between secondary inflorescence flower number and total flowers in this species, again irrespective of branch number (Fig. 2). However, the proportion of secondary to total flowers was ~66% in this case, suggesting that the secondary inflorescences share a greater proportion of the total floral potential in forget-me-not.

The unanticipated and non-intuitive floral distribution phenomenon we describe here can be rationalized in the context of the complex temporal 'decision-making' that must occur during reproductive development. In essence, the number of secondary inflorescences represents the earliest 'estimate' of the reproductive architecture the plant should produce given the available resources. However, since resource availability varies in time, a flexible system for determining flower number independently of inflorescence number allows the plant to correct for over- or underestimates of inflorescence number. This is most strikingly illustrated by floral initiation in the strigolactone mutant d14-1, which makes an erroneously high number of branches, while still initiating a wild-type number of flowers (Fig. 1E, F). Indeed, the ability to flexibly alter reproductive effort amongst synchronously-activating inflorescences might be the selective advantage that promoted the evolution and maintenance of racemic/indeterminate inflorescences over more determinate inflorescence types. While shoot branching is typically considered a binary process in which branches are either fully inhibited or fully active[3], our data suggest that, at least in the case of inflorescences, branches may continue to exert considerable influence on each other's growth after activation. We hypothesise that these observations can be explained by extension of the canalization model for apical dominance/shoot branching, in which auxin exported from actively growing branches is proposed to act via the self-organizing properties of the auxin transport system to inhibit canalized auxin export from new branches, thereby inhibiting their growth[4-6]. If

inflorescence meristem activity (and thus floral initiation rate) is regulated by the ongoing ability of inflorescences to export auxin, and if inflorescences continuously compete, via the self-organizing properties of the auxin transport system, to export their auxin into a shared stem, then the floral distribution rule could well be an emergent property of the same fundamental canalization mechanism[4,5]. Identifying the mechanism underlying the floral distribution rule will be key to understanding the generalizability and effects of the floral distribution rule among flowering plants.

METHODS

Plant materials

The following species were used for this work; Arabidopsis thaliana (Arabidopsis; Col-0, Ler, Ws-2 ecotypes), Brassica napus var. annuis (Spring oilseed rape 'Heros'), Brassica rapa (var. ZBC 005), Cardamine hirsuta (Oxford ecotype), Capsella rubella and Capsella grandiflora. The following Arabidopsis mutants were used; brc1-2[7], d14-1[8], smxl6-4 smxl7-3 smxl8-1 ('smxl678')[9], arr3,4,5,6,7,15 ('arr-*hex*')[10], arr1-4[11], gai-t6 rga-t2 rgl1-1 rgl2-1 rgl3-1 ('della')[12], gai[13]. Myosotis arvensis plants were collected from the wild on May 7th 2019, in York (UK).

Plant growth conditions

All plants were grown on Petersfield Growing Mediums No.2 Potting Supreme compost under a standard 16 h/8 h light/dark cycle (20ºC), primarily in controlled environment rooms with light provided by white fluorescent tubes at intensities of ~120µmol/m^2m^{-1}. Oilseed rape was grown under sodium lamp at an average intensity of ~250µmol/m^2s^{-1}.

Experimental design and statistics

Data in this study were gathered from a large number of independent experiments, in which each sample was a distinct plant, as described in figure legends. All data were tested for normality before statistical tests were applied. Statistical parameters are described in figure legends.

Phenotypic assessments

We assessed the numbers of flowers and branches at initial floral arrest in most species. In Capsella grandiflora, the lack of self-pollination prevents normal floral arrest, so assessments were made at the same time as for Capsella rubella. Myosotis arvensis plants of similar developmental stage were collected from the wild on 7/5/2019, and assessed at that point. Visual assessments were carried out

to record the number of each class of inflorescence, and the number of floral nodes initiated on each inflorescence. All nodes where a flower had been present were counted, regardless of whether a successful fruit had been produced or not. In terms of nomenclature, the primary inflorescence (PI) is the first bolting stem, originating from the primary (i.e. embryonic) shoot meristem. Secondary inflorescences are those initiated in the axils of primary leaves (i.e. those produced by the primary SAM). In Arabidopsis, Cardamine and Capsella, these may either be cauline or rosette, depending on the position of the parent leaf, but were treated equally in our analyses. Any inflorescences growing directly from a secondary inflorescence was classed as a tertiary inflorescence, and so forth (see Fig. 1A).

Data availability statement

All data associated with this study are presented in the figures. Data are available on request without restriction from Tom Bennett (t.a.bennett@leeds.ac.uk).

ACKNOWLEDGEMENTS

The Cardamine hirsuta seed were the kind gift of Angela Hay, and the Capsella rubella and C. grandiflora seed were the kind gift of Michael Lenhard.

AUTHOR CONTRIBUTIONS

CHW and TB designed and performed experiments, collected and analysed data and wrote the manuscript.

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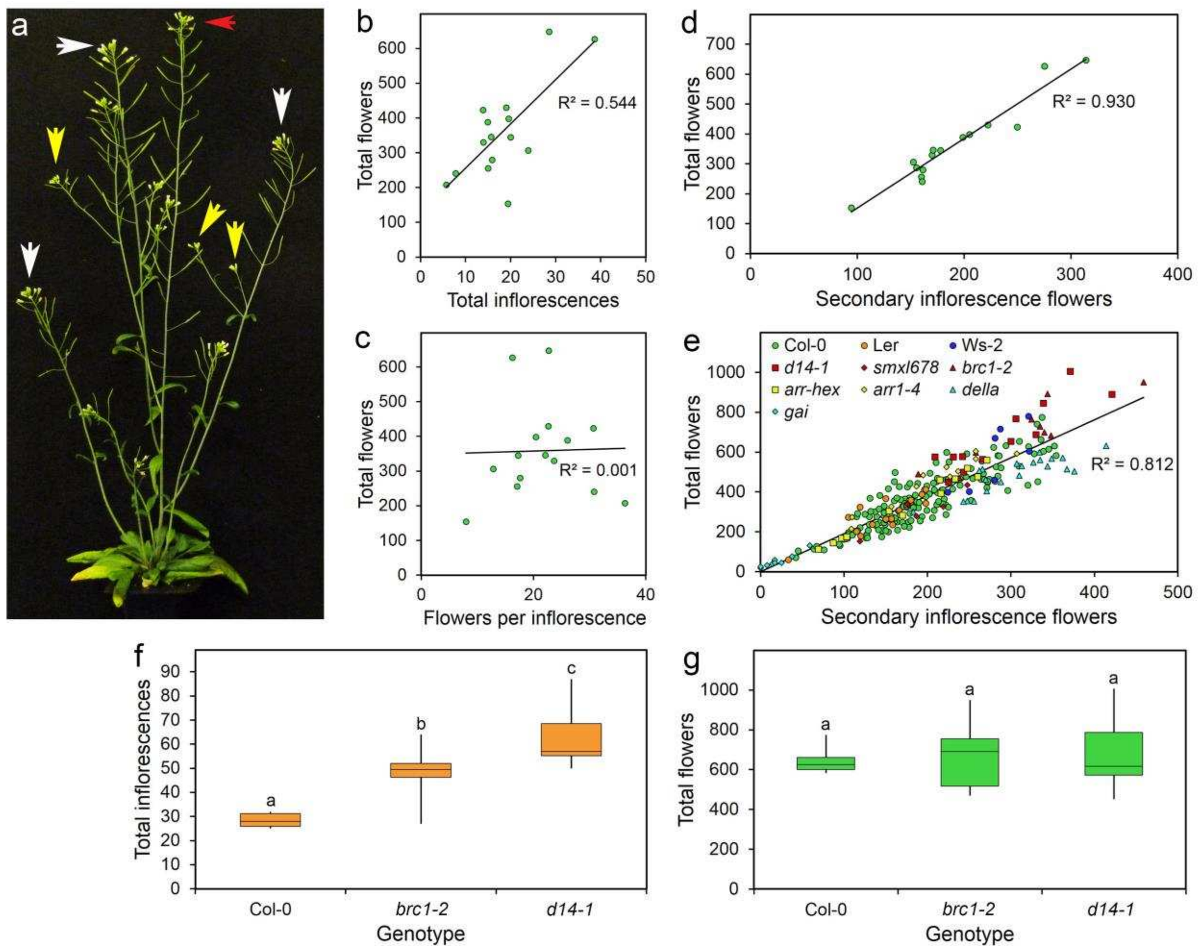

Figure 1: Flower number is regulated independently of inflorescence number in Arabidopsis

a Reproductive architecture in Arabidopsis Col-0 wild type. Arrows indicate different classes of inflorescences (red = primary inflorescence, white = secondary inflorescences, yellow = tertiary inflorescences). Secondary inflorescences include both cauline and rosette inflorescences. Following the end of flowering, each floral node (typically supporting a fruit/silique) was counted, to give the number of flowers per individual inflorescence.

b-d Graphs showing the relationship in Arabidopsis Col-0 wild-type between mean total flowers and (**b**) mean total inflorescences, (**c**) mean number of flowers per inflorescence, (**d**) mean number of flowers produced on the secondary inflorescences. n=15 independent experiments. Line of best fit was calculated by the least squares approach.

e Graph showing the relationship between total flower number and secondary inflorescence flower number in individual plants from 17 experiments, including Col-0 (n=161 independent samples),

Ler (n=18 independent samples), Ws-2 (n=7 independent samples), d14-1 (n=12 independent samples), smxl678 (n=6 independent samples), brc1-2 (n=10 independent samples), arr-hex (n=12 independent samples), arr1-4 (n=11 independent samples), della (n=21) and gai (n=9 independent samples). Line of best fit was calculated by the least squares approach across all data.

f, g Box plots showing total number of inflorescences (**f**) and flowers (**g**) produced by WT (Col-0) (n=8 independent samples) and two branching mutants (brc1-2 (n=10 independent samples), d14-1 (n=12 independent samples)) in a single experiment. The mid-line represents the median, the box the inter-quartile range, and the whiskers the maximum and minimum. Samples with the same letter are not statistically different from each other (ANOVA+Tukey HSD). Inflorescence number is significantly higher in brc1-2 (P=0.000) and d14-1 (P=0.000) than in WT (95% confidence interval; F=31.589; d.f.=2). Flower number is not statistically different in brc1-2 (P=0.917) or d14-1 (P=0.924) compared to WT (95% confidence interval; F=0.096; d.f.=2).

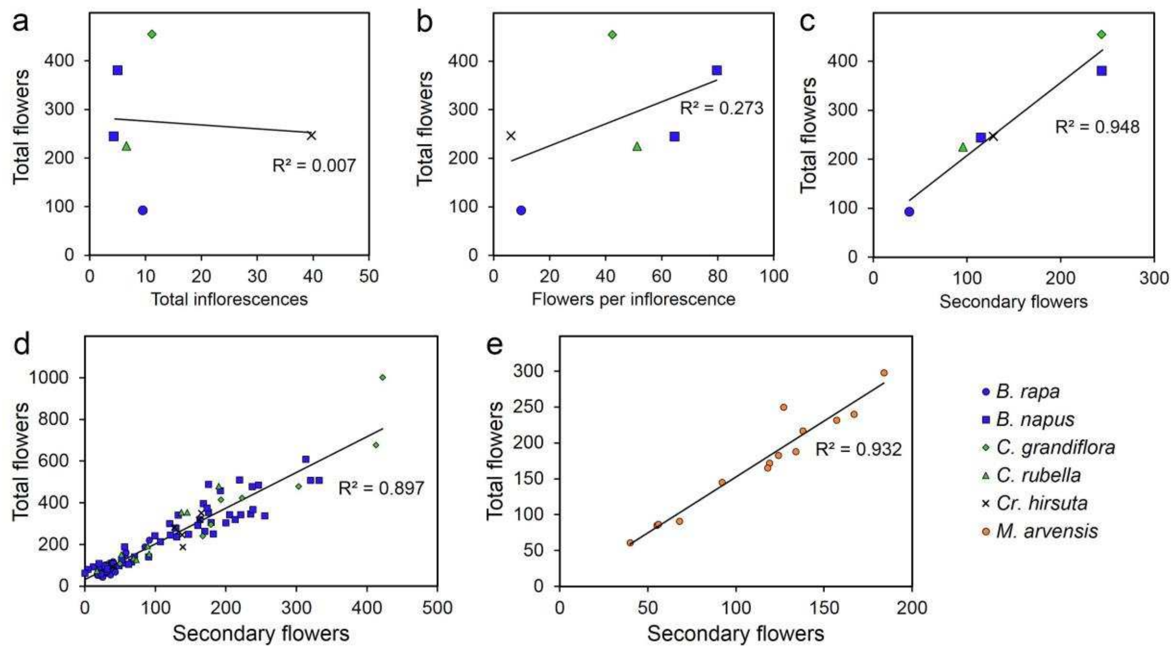

Figure 2: A conserved floral distribution mechanism regulates floral initiation across the Brassicaceae and beyond

a-c Graphs showing the relationship between mean total flowers and (**a**) mean total inflorescences (n=6 independent experiments), (**b**) mean number of flowers per inflorescence (n=6 independent experiments), (**c**) mean number of flowers produced on the secondary inflorescences in different Brassicaceae species (n=6 independent experiments); Brassica rapa, B. napus, C. grandiflora, C. rubella or Cardamine hirsuta. Line of best fit was calculated by the least squares approach across all data.

d Graph showing the relationship between the total number of flowers and the secondary inflorescence flower number in individual plants from B. rapa (n=31 independent samples), B. napus (n=49 independent samples), C. grandiflora (n=8 independent samples), C. rubella (n=9 independent samples) or Cr. hirsuta (n=6 independent samples). Line of best fit was calculated by the least squares approach across all data.

e Graph showing the relationship between the total flower number and secondary inflorescence flower number in a non-Brassicaceae species, Myosotis arvensis (forget-me-not) (n=14 independent samples).