HortScience 59(4):465–470. 2024. https://doi.org/10.21273/HORTSCI17652-23

The *Short Inflorescence* Mutation in Diploid Strawberry *Fragaria vesca* Affects Inflorescence Architecture and Runner Elongation

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Keywords. ethyl methanesulfonate mutagenesis, microscopy, peduncle elongation, runner internode lengths, single gene

Abstract. **Mutants are useful for determining the genes that underlie a given trait. This information is highly useful for developing molecular markers for breeding and is the foundational knowledge required for future genomic crop improvements. The dessert strawberry, *Fragaria* ×*ananassa*, is a valuable crop with high potential for increased use in controlled environment agriculture. The genome of the woodland strawberry *Fragaria vesca* is the dominant genome of the four diploid strawberry subgenomes that contribute to the octoploid *F.* ×*ananassa* genome. *F. vesca* is therefore a useful reference system for determining gene function and should be a useful source of gene diversity for breeding of *F.* ×*ananassa*. Chemical mutagenesis of the inbred *F. vesca* line H4 F7-3 resulted in one M2 line with a smaller stature overall and which produces flowers on very short peduncles close to the crown. This line was named *short inflorescence* (*sin*). The *sin* phenotype results from a single gene recessive mutation that is pleiotropic in that the mutation also affects internode lengths of runners as well as petiole elongation of *sin* plants. Microscopic characterization revealed that *sin* peduncles are most likely short because of a failure of cells to elongate. Inflorescences, runners, and petioles of *sin* plants were found to elongate in response to exogenous gibberellin, indicating that *sin* could be a gibberellin biosynthesis or transport mutant. A brief characterization of *sin* plants is presented to facilitate collaborative studies of the line.**

Mutants in reference plant systems have been profoundly important for identifying genes involved in developmental processes (e.g., Smyth 2023). The diploid woodland strawberry, *Fragaria vesca*, has many features that make it an attractive system for identifying genes important for development in a perennial plant, including: small size; small (~240 Mb) well-sequenced and characterized genomes (Edger et al. 2018; Shulaev et al. 2011; Zhou et al. 2023); other closely related wild diploid strawberry species found throughout the world in the temperate zones (Darrow 1966); an edible aromatic organ known as the berry, which is important for sexual reproduction; and facile clonal vegetative reproduction through overground stems, stolons, commonly called runners. Several transformable inbred lines of *F. vesca* are available (Slovin and Rabinowicz 2007; Slovin et al. 2009), and chemical mutagenesis of these lines resulted in mutations affecting such traits as runner production (Caruana et al. 2018; Li et al. 2018) and fruit shape (Wang et al. 2017), as well as leaf and flower development (Han and Kang 2023). Numerous genomics and genetic tools are available at the Genome Database for Rosaceae, https://www.rosaceae.org/ (Jung et al. 2019).

In the genus *Fragaria,* multiple flowers are produced on an inflorescence that has historically (Darrow 1966) been classified as a cyme. Recent analysis of the woodland strawberry inflorescence architecture (Lembinen et al. 2023) reclassified the inflorescence structure as that of a closed thyrse; defined therein as a "compound inflorescence with determinate primary monopodial axis and lateral sympodial branches." For commercial purposes, the more desirable inflorescence is one that is long enough to extend outside of the foliar canopy to facilitate pollination and harvesting, as well as minimize disease pressure.

Due to its polyploid genome, *F.* ×*ananassa* is primarily produced commercially from clonal plants that develop at the tip of the runners. The development and elongation of the runner is dependent on the production of gibberellin, as mutation of a gene involved in gibberellin biosynthesis, gibberellin$_{20}$oxidase 4 (*FveGA20ox4*) abolishes runner production (Tenreira et al. 2017) and mutation of the DELLA transcription factor FveRGA1, a repressor of genes whose transcription is induced by gibberellin, restores runnering of the naturally occurring *FveGA20ox4* mutant (Caruana et al. 2018; Li et al. 2018).

Strawberry runner growth from the meristem requires both cell division and cell elongation, which first result in a usually nonproductive node, although the node occasionally produces a thin, weak runner (Darrow 1929, 1966; Guan et al. 2019). Following further extension, a clonal daughter plant capable of forming its own runners is produced at the tip. This daughter plant has root initials that respond to moisture by forming roots. Thus, growth of the two internodes (I-1 or proximal and I-2 or distal) results in two different outcomes, one of which is crucial to vegetative clonal propagation.

Gibberellins affect many important traits in horticultural crops (Zhang et al. 2022). Differences in elongation growth patterns were observed between *F.* ×*ananassa* runner I-1 and I-2 (Nishizawa and Hori 1993), although runner length overall increases in response to gibberellin (Leshem and Koller 1966). The lengths of I-1 and I-2 of *F.* ×*ananassa* runners are variable, but are generally equal (Darrow 1929). Peduncle length was also found to increase in response to gibberellin, as does petiole length (Guttridge and Thompson 1963; Leshem and Koller 1966). Photoperiod also affects runner and peduncle elongation (Nishizawa 1994a, 1994b).

EMS mutagenesis of H4 F7-3, an inbred line of *F. vesca* (Slovin and Dougherty 2023) resulted in one M2 line that is shorter in stature, with inflorescences and runners that are also distinctly shorter than wild type (WT) (Fig. 1). This mutant was first identified because the open flowers were found close to the crown of the plant on short peduncles and was named *short inflorescence* (*sin*). We describe here a preliminary characterization of this mutant so as to further collaboration on the molecular nature of the mutation and the understanding of organ elongation in *Fragaria* and other members of the Rosaceae family.

Materials and Methods

Plant material and growth conditions. Plants of *F. vesca* L. subsp. *vesca* forma *semperflorens* (Duchesne) Staudt inbred line Hawaii4 F7-3 (H4F7-3), PI664444, and the EMS mutant *short inflorescence* (*sin*) were grown in the greenhouse or growth chamber under a long-day photoperiod essentially as described in Slovin and Dougherty (2023). Seeds were collected using the blender method (Morrow et al. 1954). The mutant was identified by visual screening of more than 3000 M2 plants derived from EMS-treated seed (Slovin and Dougherty 2023). The mutant was propagated vegetatively by separating branch crowns or pegging runner plants. Branch crowns or pegged runners were rooted on a mist bench

Received for publication 5 Dec 2023. Accepted for publication 15 Jan 2024.
Published online 29 Feb 2024.
Sam Jones is thanked for help with the mutagenesis.
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for at least 2 weeks, then transferred to the greenhouse or growth chamber.

Inflorescence lengths were measured from point of attachment to the base of the first opened flower once all flowers on that inflorescence had opened on 18 WT (Hawaii 4 F7-3) plants and 16 *sin* plants grown from runner plants of the same age. Runners were measured for total length once two daughter plants had developed and the runner developing on the second plant was just visible. These measurements were made on runners from 18 plants with WT phenotypes and eight plants with the *sin* phenotype from a segregating F2 population. The F2 population was produced by backcrossing *sin* to WT to produce an F1, then selfing one F1 plant to produce the seed for the F2 population. The ratio of length of the first internode (I-1) to the length of the second internode was determined once the daughter plant at the tip of the runner had one unfolding trifoliate leaf. Statistics used the Student's *t* test and χ^2.

Microscopy. Peduncle, pedicel, and runner segments from *sin* and WT plants were fixed in FAA (formaldehyde:ethanol:acetic acid:water, 10:50:5:35) and embedded in paraffin as described (Slovin and Dougherty 2023). Sections (10 μm) were stained with Safranin O and Fast Green, and slides were examined under bright field using a Zeiss Axio Zoom V16 and photographed using Zeiss Zen 3.6 (blue edition) software. Measurements were made on at least four sections from at least three biological samples of peduncles from inflorescences on which the primary flower was just fully open. Measurements were determined to be significantly different using the Student's *t* test.

Gibberellin treatment. Individual branch crowns from WT and *sin* plants were rooted under a mist bench, then placed in a growth chamber and grown for 1 week. Then, visible inflorescences and runners were removed, and crowns trimmed back to one fully expanded leaf and one visible but still folded leaf before spraying with 10 mM GA_3 in 1.0% ethanol in H_2O. Control plants were sprayed with 1.0% ethanol in H_2O. Plants were sprayed to runoff once per week for 2 weeks and imaged 1 month later.

Results

Plant description. The M2 mutant plant EMS-068, was named *short inflorescence* (*sin*) because it was initially identified due to its short stature and short inflorescences as compared with WT (Fig. 1A). Subsequently, the mutant plants were also found to produce short runners (Fig. 1B) with short internodes. Flowers on young plants were never seen above the canopy because buds matured and opened on the very short peduncles (Fig. 1D), unlike WT, where the peduncles elongated so that open flowers were found at or above the leaves (Fig. 1C). Flower buds on the first *sin* plant inflorescence open when the bud is still close to the crown (Fig. 1E). The peduncles on later inflorescences of *sin* plants elongate (Fig. 1F) but remain shorter than those of WT plants. Although the flowers of *sin* plants are smaller than WT (Fig. 1G), bracts, sepals, and petals of the mutant flowers appear normal in morphology, and pollen is produced.

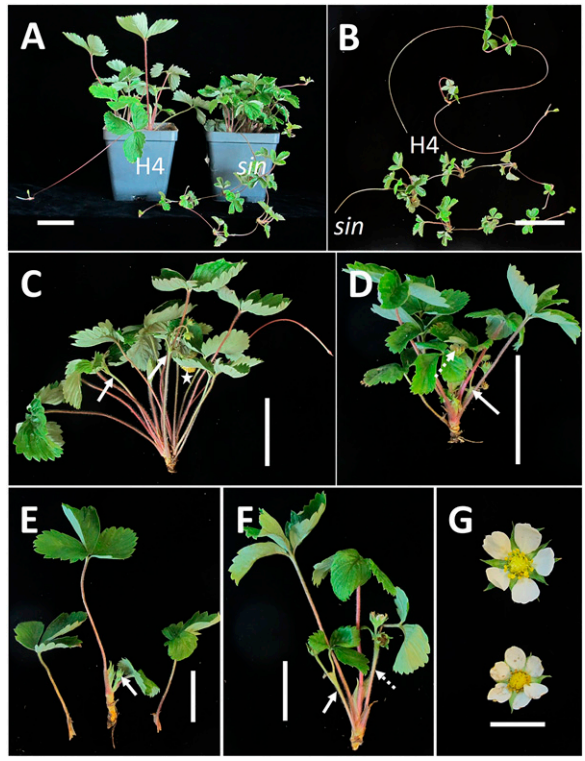


Fig. 1. Morphology and physiology of diploid strawberry Hawaii 4 (wild-type) and the *short inflorescence* mutant. (**A**) Wild-type (H4) are taller than *short inflorescence* (*sin*) plants. Bar = 5.0 cm. (**B**) Internodes on H4 runners are longer than those on comparable aged *sin* runners. H4 and *sin* demarcate the point of attachment to the mother plant. Bar = 5.0 cm. (**C**) and (**D**) Crowns from H4 and *sin* plants of the same age, each consisting of more than one branch crown. Solid arrows point to inflorescences. The dotted arrow indicates an open flower. The asterisk indicates a young developing fruit. Bars = 5.0 cm. Branch crowns from a single *sin* plant illustrating a very short inflorescence (**E**) and the longest inflorescence on the same plant (**F**). On further dissection, bud at arrow in (**E**) was determined to be at early stage 12 (Hollender et al. 2012). In (**F**), the bud on the inflorescence indicated by the solid arrow was also at stage 12, whereas one flower was open and one senescing on the inflorescence indicated by the dotted arrow in (**F**). Bars in (**E**) and (**F**) = 2.0 cm. (**G**) Open flowers of H4 (top) and *sin* (bottom) plants. Bar = 1.0 cm.

Genetics. Inflorescence lengths, as measured from point of attachment to the crown to the base of the first open flower, were significantly shorter for *sin* plants than WT (Fig. 2A). Like WT, *sin* plants produce berries if manually pollinated with WT pollen. All F1 plants produced from reciprocal backcrossing

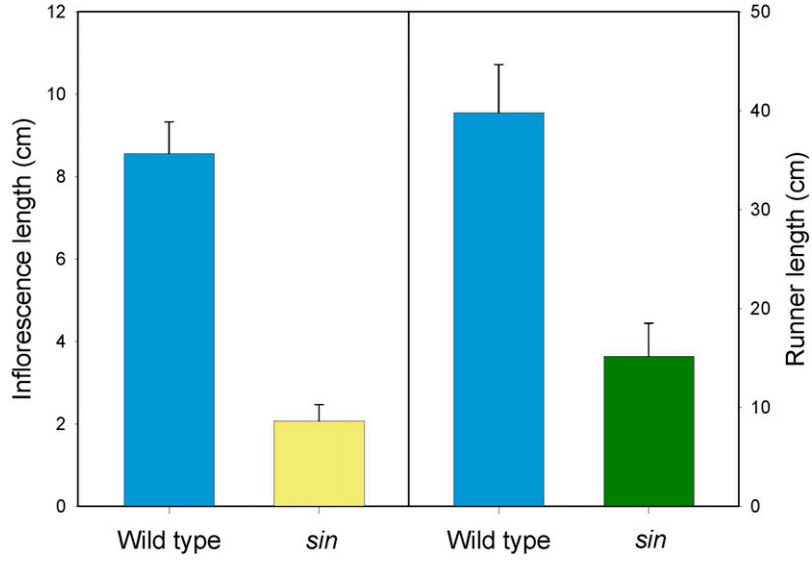


Fig. 2. Inflorescence lengths (**A**) and runner lengths (**B**) from wild-type (WT) and *sin* plants in an F2 population developed by crossing *sin* by Hawaii 4. The average lengths of *sin* inflorescences and runners were significantly shorter than those of WT. Error bars represent the average ± *SD* of ≥10 measurements.

of *sin* and WT had the WT phenotype. Seventy-five F1 seeds were sown, of which 62 germinated, resulting in 62 F2 plants that were scored as mutant or WT dependent on inflorescence and runner phenotypes. Flowers appeared on WT plants and *sin* plants in the F2 population at almost the same time, with the first open flower on a WT plant appearing 2 d before the first open flower on a *sin* plant. The average length of the first inflorescences from the first 18 WT plants to flower and the first 16 *sin* plants to flower are shown in Fig. 2. Inflorescences from *sin* plants are significantly shorter than WT. The average length of 30 runners from WT F2 plants and from eight *sin* plants (Fig. 2) shows that *sin* runners are significantly shorter than WT runners. Runners were measured when leaves of the second daughter plant had just started to unfold.

The distribution of lengths of first inflorescences from all F2 plants is shown Fig. 3A. Data show that inflorescence length is a quantitative trait with lengths from WT F2 plants ranging from 3.4 cm to 10.9 cm and inflorescence on *sin* plants ranging from 1.7 cm to 3.2 cm. The average inflorescence length of six WT plants was 7.2 cm, with lengths ranging from 5.2 cm to 8.5 cm.

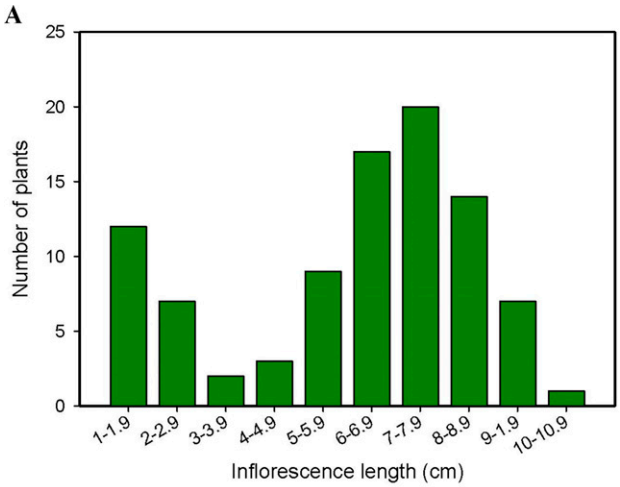
The *sin* phenotype was found for 18 of the 62 F2 plants, indicating that the *sin* phenotype results from a recessive mutation in a single gene (Fig. 3B). The *sin* mutation appears to have pleiotropic effects, affecting runner length (Fig. 2B) and petiole length (Fig. 1A) as well as inflorescence length. The 18 *sin* F2 plants all had runners with short internodes as well as shorter inflorescences. All 18 *sin* F2 plants were shorter in stature than the 44 F2 plants scored as having a WT phenotype.

Cell length. Longitudinal thin sections (10 μm) of peduncles from mature inflorescences, stained with Safranin O and Fast Green, revealed that, in general, cortical cells of *sin* peduncles are shorter in length than cortical cells of WT peduncles (Fig. 4A). Cell measurements were made along contiguous files of cells at three different positions in at least four serial sections per slide, each slide coming from an inflorescence from three separate plants. To capture the maximum extent of variation, files of cells were measured on both sides of the vasculature. WT peduncles averaged 13.9 cells per mm, or 72 μm per cell. Mutant peduncles averaged 20.9 cells per mm, or 48 μm per cell (Fig. 4C).

Cross sections through WT and *sin* peduncles (Fig. 4B) revealed that cortical cells varied widely in diameter in both genotypes; however, there were no obvious differences in the pattern of development of the stele (Fig. 4C). Although no measurements were made, pith cells in the cross sections appear to be of similar diameter in both genotypes, as do epidermal cells. Large multicellular trichomes are visible in the epidermis of both genotypes. In the sections shown in Fig. 4B, there appears to be enhanced development of sclerenchymous fibers between the endodermis and the phloem in the *sin* peduncle. This phloem fiber region stains differently from the surrounding cells and is visible to some degree in all the *sin* sections observed.

Runner structure. When measuring runner lengths for Fig. 2B, it was noted that some runners from *sin* plants did not appear to have the expected structure consisting of I-1, a node, I-2, then daughter plant. These *sin* runners appeared to be missing the node as well as an I-2, although a daughter plant had developed (Fig. 5A). Figure 5A shows the node and I-2 of the first daughter plant (1), and the I-1 of the first runner produced by that daughter plant. The runner was photographed 5 d after I-1 was marked at 0.5-cm intervals when the runner tip, which would subsequently develop into plant 2, was a tight bud. During the 5-d interval, growth occurred primarily at the proximal end of I-1.

I-1 and I-2 were measured on runners from plants with *sin* and WT phenotypes in the F2 population. F2 *sin* runners were significantly shorter than those of F2 WT runners and Hawaii 4 (WT control) runners of the same age (Fig. 5). Unlike F2 WT and Hawaii 4 controls, overall the I-2 of *sin* plants were always shorter than I-1 (Fig. 5B) and in some cases, as shown in Fig. 5A, the node and I-2 appeared to be nonexistent. I-1 and I-2 of Hawaii 4 and WT runners are essentially equal in length (Fig. 5B).



B

Phenotype	Expected	Observed	X^2	P range
Wild-type	46.5	44	0.134	
short inflorescence	15.5	18	0.403	
Total		62	0.537	>0.05

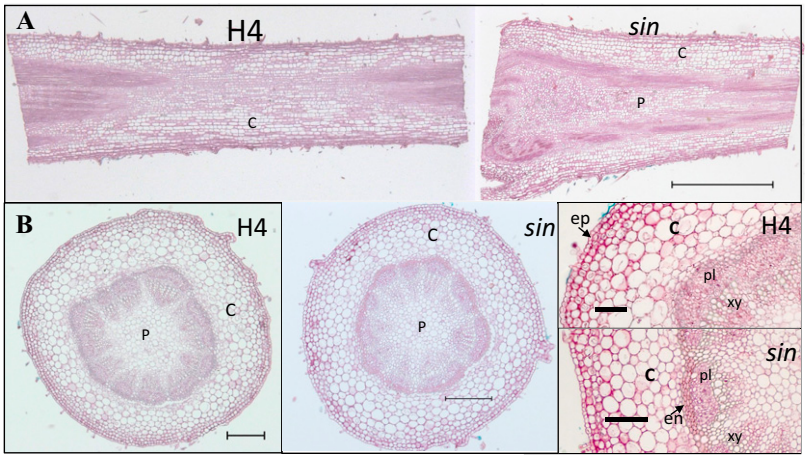
Fig. 3. Genetics of the *sin* mutation. An F2 population of 62 plants was scored for length of the first inflorescence at the time of first flower opening (**A**). Inflorescence lengths of wild-type (H4) plants ranged from 5.1 to 10.7 cm. (**B**) Chi-squared analysis indicates that a single recessive mutation results in the *sin* phenotype.

Discussion

The architecture of the strawberry inflorescence determines the numbers of flowers and thereby the number and size of fruit produced, how easily the flowers can be pollinated by insects, and how easily the fruit can be picked. Commercial berry harvest is still primarily done manually. A recent study of inflorescences from *F. vesca* revealed that the variety of branching observed is regulated by homologs of the Arabidopsis *TERMINAL FLOWER 1* and *FLOWERING LOCUS T* (Lembinen et al. 2023). Branching affects the number and size of berries, whereas longer and stronger peduncles are desirable for berry production outside the plant canopy, where the berries are more accessible to rapid manual or robotic picking and flowers are more accessible to pollinators.

The dessert strawberry, *F.* ×*ananassa* is a high-value commodity with an octoploid genome. Of the four diploid strawberry subgenomes found in the octoploid, the *F. vesca* genome appears to be dominant (Edger et al. 2019; Feng et al. 2021). We identified a mutant of *F. vesca* that has very short peduncles, resulting in flowers that open within the canopy close to the plant crown (Fig. 1). We further characterized the mutant, which has the potential to allow us to identify genes that regulate or participate in inflorescence elongation.

We found that the *sin* short phenotype results from a recessive mutation of a single gene (Fig. 3), and that this mutation is pleiotropic in that plants in an F2 population that were scored as having short inflorescences also exhibited short runner internodes, resulting in short runners with multiple daughter plants (Fig. 1). The short inflorescence may result in part from failure of cortical cells in the *sin* inflorescence to elongate to the same extent as WT inflorescence cells (Fig. 4). Although measurements were not made from cross sections, our observations indicate that cortical cells in *sin* inflorescences appear to be smaller in diameter than those in WT (Fig. 4B). Based on counts of peduncle epidermal cells, Nishizawa (1994b) concluded that *F.* ×*ananassa* peduncles were longer



C

	Number of measurements	Number of cells/mm ± SD
Wild-type	38	13.9 ± 2.8
short inflorescence	48	20.9 ± 3.9

P <0.001

Fig. 4. Longitudinal sections (**A**) and cross sections (**B**) of peduncles from Hawaii 4 (WT) and mutant (*sin*) plants. (**A**) c denotes cortex, p denotes pith. Bar = 1.0 mm. (**B**) Left two panels: c denotes cortex, p denotes pith. Bars = 100 um. (**B**) Right two panels: c denotes cortex, ep denotes epidermis, e denotes endodermis, pl denotes phloem, xy denotes xylem. Bars = 50 um. (**C**) The number of cortical cells per mm in peduncles of WT and *sin* plants. Measurements were made from serial sections of peduncles from three plants of each genotype. The number of cells/mm was significantly higher (P <0.001) in *sin* sections than in WT.

in length under long-day photoperiods than under short days, primarily because of a greater number of cells rather than an increase in cell length. Thus, the shortened inflorescences, runners, and peduncles found on *sin* plants could also result from decreased cell division, and this will need to be investigated further.

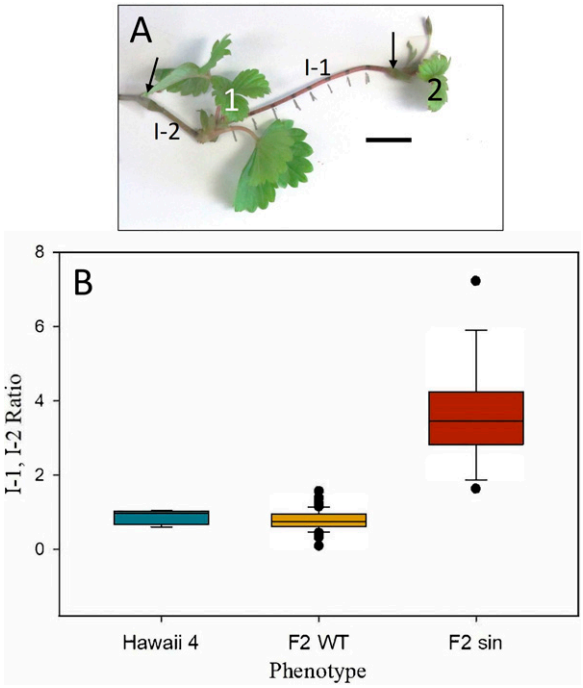


Fig. 5. The internodes of runners from *sin* plants are not equal in length. (**A**) The node on this *sin* runner, which is the first runner produced by daughter plant 1, is directly behind daughter plant 2, so the length of Internode 2 (I-2) for this runner would be scored as zero. The photograph was taken 5 d after Internode 1 (I-1) was marked at 0.5-cm intervals. The positions of the markings are indicated by perpendicular lines below the runner. Arrows indicate the nodes. (**B**) The ratio of the length of I-1 to I-2 for runners from Hawaii 4 wild-type (WT) plants (n = 5); plants scored as WT from the F2 population described in Fig. 3 (n = 37); and plants scored as *sin* from the same F2 population (n = 10). Two additional measurements from *sin* plants were scored with I-2 = 0.

In our samples, obvious cell size differences were sometimes noted for cortical cells from one side of the peduncle to the other, possibly related to the amount of light reaching one side vs. the other (see for example, the *sin* specimen in Fig. 4). To capture the maximum extent of variation, we made three or more measurements of cortical cell numbers per given length along cell files at various positions on each section of at least four serial sections from each biological replicate. We concluded that cortical cells from *sin* peduncles are significantly shorter than those from WT peduncles (Fig. 4C). The peduncle is composed of several cell types, from the surface epidermis, through the parenchymous cortex, the phloem, and the xylem to the central, parenchymous, pith. Sachs (1965) wrote that, in many cases, the rate of cell division is greater in cortical cells than in the pith. Our conclusion that the *sin* mutation primarily affects cell length results only from observing cortical cells, so it remains to be determined if the *sin* mutation affects cell division as well.

The effect of the mutation on runner elongation is more striking for the second internode than for the first. For some runners, internode 2 is so short that it is not visible (Fig. 5). The differential internode growth could reflect the lack of a growth factor such as gibberellin reaching the second internode from the mother plant, either because of decreased biosynthesis or decreased transport. Alternatively, cells of the second internode in *sin* plants may be unable to sense or respond to an elongation promoting signal, possibly resulting from failure to express a specific GID receptor. An early proteomics study (Fang et al. 2011) described the major proteomic differences between the proximal internode (I-1) and the distal internode (I-2); however, the technique used would not have identified regulatory factors directing the different developmental outcomes. A more recent proteomics analysis of runner nodes identified 7271 proteins and revealed distinct protein differences among the tissues examined: the dormant axillary bud at node 1; the runner apical meristem; the runner producing axillary bud at node 2; and the developing leaves at node 2 (Guan et al. 2019). However, no mechanism(s) for the developmental differences at node 1 and node 2 were obvious from the data that would account for the phenotype of the *sin* runners.

Application of gibberellin to *F.* ×*ananassa* plants at flower differentiation increased peduncle elongation as well as the length and number of subsequent runners (Leshem and Koller 1966). In contrast, an inhibitor of gibberellin biosynthesis, (2 chloro-ethyl) trimethylammonium chloride inhibited peduncle and runner elongation, as well as the number of runners produced (Leshem and Koller 1966). These experiments suggested that the mutation in *SIN* might be affecting gibberellin biosynthesis, or the ability of *sin* cells in the inflorescence and runners to respond to gibberellin.

As a preliminary observation, we treated 10 plants of each genotype with GA_3, and 10 plants of each genotype served as controls.

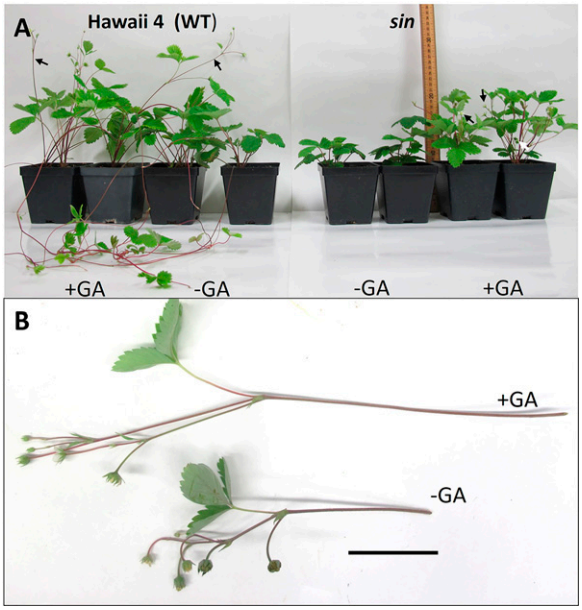


Fig. 6. Wild-type (WT) and *sin* plants respond to treatment with gibberellin A_3 (GA). (**A**) Four exemplary plants of each genotype are shown; two were treated with GA (+) and two were treated with control solution (-). The ruler in the background measures centimeters. Black arrows point to open flowers at or above canopy height. The white arrow points to flowers that opened close to the crown because they were on a short peduncle. (**B**) Closeup of WT inflorescences treated (+GA) or not (-GA) with gibberellin when the inflorescence was differentiated in the crown but before the inflorescence had become visible. Bar = 5.0 cm.

WT *F. vesca* plants (Fig. 6A) showed a clear response to exogenous gibberellin, producing highly elongated inflorescences (Fig. 6B) and runners. Mutant plants also respond to gibberellin. Petioles of *sin* plants elongated in response to GA_3 treatment, resulting in an overall taller plant. Peduncles of *sin* plants also responded to GA_3 treatment, as open flowers were observed at canopy height due to elongation (Fig. 6A, black arrows). Unlike what was observed for WT, some *sin* inflorescences did not elongate (Fig. 6A, white arrow). Although all visible inflorescences were removed from plants before treatment, it is possible that because it is short, the *sin* inflorescence may be more developmentally advanced than WT before it becomes visible. The cells in these *sin* peduncles may no longer be able to fully respond to GA, similar to what was observed for internode 2 of *sin* runners.

Alternatively, our results can be interpreted to indicate that the *sin* mutation does not directly affect inflorescence and runner length through gibberellin biosynthesis, transport, sensitivity, or response. Identification of the mutated gene through bulked sequencing analysis (e.g., Abe et al. 2012) could point more directly to how the *sin* mutation produces this pleiotropic response involving runnering and flowering.

WT, mutant plants, and F1 seeds produced from backcrossing *sin* to H4F7-3 are available to the community for further collaborative research. Please contact Janet Slovin or the US Department of Agriculture Agricultural Research Service National Clonal Germplasm Repository for materials.

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