Strigolactones promote flowering by inducing the miR319-LA-SFT module in tomato

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Strigolactones promote flowering by inducing the miR319-*LA*-*SFT* module in tomato

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Strigolactones are a class of phytohormones with various functions in plant development, stress responses, and in the interaction with (micro)organisms in the rhizosphere. While their effects on vegetative development are well studied, little is known about their role in reproduction. We investigated the effects of genetic and chemical modification of strigolactone levels on the timing and intensity of flowering in tomato (*Solanum lycopersicum* L.) and the molecular mechanisms underlying such effects. Results showed that strigolactone levels in the shoot, whether endogenous or exogenous, correlate inversely with the time of anthesis and directly with the number of flowers and the transcript levels of the florigen-encoding gene *SINGLE FLOWER TRUSS* (*SFT*) in the leaves. Transcript quantifications coupled with metabolite analyses demonstrated that strigolactones promote flowering in tomato by inducing the activation of the microRNA319-*LANCEOLATE* module in leaves. This, in turn, decreases gibberellin content and increases the transcription of *SFT*. Several other floral markers and morpho-anatomical features of developmental progression are induced in the apical meristems upon treatment with strigolactones, affecting floral transition and, more markedly, flower development. Thus, strigolactones promote meristem maturation and flower development via the induction of *SFT* both before and after floral transition, and their effects are blocked in plants expressing a miR319-resistant version of *LANCEOLATE*. Our study positions strigolactones in the context of the flowering regulation network in a model crop species.

flowering | *LANCEOLATE* | miR319 | strigolactones | tomato

Significance

We report that the phytohormones strigolactones promote tomato flowering. Our data suggest that this occurs via transcription of the florigen-encoding *SINGLE-FLOWER TRUSS* (*SFT*) gene in the leaves. *SFT* transcription is linked upstream to transcriptional reprogramming including increased levels of miR319 and decreased transcripts of its *LANCEOLATE* target, a repressor of *SFT* transcription, in the leaves and meristems. A higher content of gibberellins is also likely to contribute to the poor reproductive performance of strigolactone-depleted tomato. Our study opens novel opportunities to manage fruiting time and total yield for this crop.

The switch from the vegetative to the reproductive phase is called floral transition and is characterized by the production of flowers instead of leaves by the shoot apical meristem. In plants such as the day-neutral *Solanum lycopersicum* (tomato), shoot apical meristems robustly transition to flowering after producing six to nine leaves, depending on the cultivar. Following transition, flower buds enter flower development, the rate of which contributes to defining the timing and intensity of flowering in the plant. The right timing of this transition and of flowering itself plays a pivotal role in the plant life cycle: It is a prerequisite for successful reproduction and environmental adaptation, upon which plant survival depends. Floral transition and flower development are also crucial variables for productivity in fruit and grain crops, with huge agronomical relevance and direct impact on yield.

Flowering is finely regulated by the interaction of multiple genetic pathways and responds both to endogenous hormonal cues and environmental signals (1). A common feature of all flowering plants is that a mobile and graft-transmissible signal is produced in the leaves and reaches the apical meristem via the phloem stream. Such signal, initially called "florigen," is now characterized and in tomato is the protein encoded by *SFT* (*SINGLE FLOWER TRUSS*), the homologue of *FLOWERING LOCUS T* (*FT*) in *Arabidopsis thaliana* (Arabidopsis) (2). Because of rising *SFT* levels, the apical meristem undergoes conversion to a transitional meristem and then to inflorescence and floral meristems (3, 4). *SFT* is also crucial for flower development (5): the tomato *sft* mutants (6) are not only late flowering but they also produce reduced inflorescences or a few flowers and then revert to vegetative functioning. The floral transition requires multiple players and complex interactions; at least five integrated flowering pathways are known in Arabidopsis, all of which affect the expression of *FT* (4, 7, 8). Contrarily to Arabidopsis, tomato floral transition is not affected by the photoperiod (3) or by vernalization (9); it is instead strongly influenced by the age-dependent pathway, by the action of gibberellins, and by a recently described pathway encompassing the microRNA miR319 (10).

The age-dependent pathway ensures that flowering takes place when the plant has accumulated enough resources to sustain it. It requires the age-dependent reduction of miR156 levels and the transcript increase of its main targets, the transcription factors

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The authors declare no competing interest.

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SQUAMOSA-PROMOTER BINDING PROTEIN-LIKE (SPLs or SBP-box, later SBPs) (11). In tomato, SBPs activate phase transition by directly inducing *SFT* in leaves and *MADS-box* genes in the shoot apical meristem (10–12). In parallel, the phytohormones gibberellins also play a key role in flowering induction, but their effects are species dependent: while they promote floral transition in Arabidopsis (13), they act as inhibitors in tomato (10, 14). Upon gibberellin perception, the DELLA proteins are degraded by the proteasome. DELLAs are key negative regulators of the gibberellin signaling pathway (15, 16) and can either activate or deactivate their targets; they also bear genetically separable roles in controlling vegetative and reproductive development (17).

The age-dependent and gibberellin pathways are integrated by the miR319 pathway. In tomato, miR319 promotes flowering by decreasing the transcripts of the TEOSINTE BRANCHED1/CYCLOIDEA/PCF (TCP) gene *LANCEOLATE* (*LA*), as confirmed by the early-flowering phenotype of *LA*-silenced plants (18) and by the delayed flowering of plants expressing a miR319-resistant version of *LA* (10). LA also increases the expression of gibberellin biosynthetic genes, decreases the expression of their catabolic genes, and thus induces higher levels of the active phytohormone, which contributes to delayed flowering in tomato (10, 19). Furthermore, LA represses *SBP* transcription, thus interacting both with the age-dependent and the gibberellin pathways. However, LA also directly inhibits *SFT* expression in leaves, and thus, miR319 can promote floral transition and flower development without the need for gibberellins or SBPs (10). Which or how other hormones may affect the miR319-LA-SFT module is currently unknown.

Strigolactones were discovered as novel carotenoid-derived phytohormones in 2008. Beyond the initial role as signaling molecules in the rhizosphere, they shape plant architecture by inhibiting axillary bud outgrowth, promoting secondary shoot growth and leaf senescence, and affecting root development (20). They are also involved in the responses to abiotic stress (20). For example, they boost antioxidant responses and modulate stomatal activity, at least partly, via cross talk with abscisic acid (ABA) and the microRNA miR156 in tomato (21–23). Finally, strigolactone mutants show general reproductive defects in several—though not all—species. For example, knocking down the biosynthetic gene *CAROTENOID CLEAVAGE DIOXYGENASE* 7 (*CCD7*) makes *Lotus japonicus* produce fewer flowers, fruits, and seeds (24). Among solanaceous plants, the most severely affected potato lines silenced for *CCD8* (encoding the dioxygenase acting downstream of CCD7) do not flower at all (25) and in petunia, delayed flowering time and smaller flowers have been reported for analogous lines (26). In tomato, *CCD8* silencing causes fewer and smaller flowers and fruits (27). So far, little effort has been put into investigating the molecular underpinnings of these phenotypes, but for the finding that auxin amounts and distribution are altered during fruit ripening in strigolactone-depleted vs. wild-type (wt) tomato (27). However, auxin levels have been found equal in flowers of wt and *CCD7*-silenced *L. japonicus* (24). Thus, the molecular mechanisms explaining strigolactone effects on flowering remain largely unclear.

Our study aimed at investigating the role of strigolactones in the molecular network regulating vegetative to reproductive phase transition and flower development in tomato, focusing mainly on the generation of the florigen signal in leaves. We assessed the developmental and molecular effects of excess or depleted strigolactone levels in the shoot, and we showed that endogenous and exogenous strigolactones promote flowering. Through transcriptomics and targeted expression analysis, we examined which functional gene families and pathways linked to flowering are regulated by these hormones. We demonstrated that strigolactones promote flowering by affecting many flowering-related genes, notably by activating the miR319-*LA*-*SFT* module in leaves and meristems, with a likely contribution by a reduction of bioactive gibberellin content.

Results

***CCD7* Transcript Levels Correlate with Flower Development and *SFT* Transcript Accumulation in Leaves.** While the vegetative and stress-related phenotype of *CCD7*-silenced tomato plants (SL- hereafter) has been described (22, 28–32), their reproductive defects have not been investigated to date. To address this point, we contrasted self-grafted SL- plants with the corresponding self-grafted wt and with heterografted plants, in which a wt scion is grafted onto a SL- rootstock (wt/SL-). The latter combination leads to a significant transcriptional activation of strigolactone biosynthetic genes in the leaves, as demonstrated earlier in tomato (32) and other species such as pea (33), and shows no obvious morphological deviation from wt plants during the vegetative phase (*SI Appendix*, Fig. S1*A*). Thus, wt/SL- plants were used here to describe the effects of increased endogenous strigolactones in a wt shoot in relation to flowering. The low strigolactone levels in the SL-/SL- self-grafted plants led to a significant decrease in the number of flowers per plant [each counted only once, at the anthesis stage (34)], statistically detectable from 35 d after grafting. Conversely, starting 25 d after grafting, the new flowers on the wt/SL- plants were more than double the number per plant compared to the self-grafted wt/wt plants (Fig. 1*A*). The number of fruits per plant collected 60 d after the grafting reflected these differences (Fig. 1*B*), while the cumulative plant yield at the end of the harvesting season did not (*SI Appendix*, Fig. S2*A*) because wt/wt plants generally produced bigger fruits. Heterografted plants also showed shorter times to anthesis than wt/wt plants (Fig. 1*C*), and the number of leaves at anthesis correlates with the timing of flowering (i.e., fewer leaves at anthesis in wt/SL- than wt/wt plants, *SI Appendix*, Fig. S2*B*). Note that grafting can hardly be performed before the early reproductive stage, when phase transition has already occurred; thus, our plants had already transitioned at grafting, so this difference can only reflect faster flower development in heterografted plants, and not faster meristem transition. Finally, *SFT* transcripts 30 d after grafting correlate positively with the transcriptional activity of the strigolactone biosynthetic pathway in leaves, since the heterografted plants displayed higher values than wt/wt, and SL-/SL- plants showed the lowest (Fig. 1*D*).

Treatment with the Synthetic Strigolactone Analogue GR24^{5DS} Promotes Flowering and Leads to *SFT* Induction in Leaves. To gain further support for the role of strigolactones in flowering, and to capture the possible modulation of floral transition, we investigated the effect of spraying plant leaves with a 5 µM solution of the synthetic strigolactone analogue GR24^{5DS} on meristem development and time to anthesis. Exogenous strigolactones appeared to accelerate the speed of meristem maturation when delivered on juvenile plants (*SI Appendix*, Fig. S3) and more so when a second treatment was delivered right after transition (Fig. 2*A*). In addition, when 3-wk-old (beginning of the reproductive phase) wt plants were treated, they brought anthesis significantly forward compared to control plants (Fig. 2*B*); a similar trend was observed on wt/wt, self-grafted plants (Fig. 1*C*) treated 25 d after grafting. As for wt/SL- plants, the number of leaves at the time of flowering tended to be lower (albeit not significantly) for GR24^{5DS}-treated than mock-treated wt/wt plants (*SI Appendix*, Fig. S2 *B* and *C*). Consistently with the previous observations on grafted plants, *SFT* transcripts increased in leaves to become significantly higher than the mock-treated control 24 h after GR24^{5DS} treatment (Fig. 2*C*).

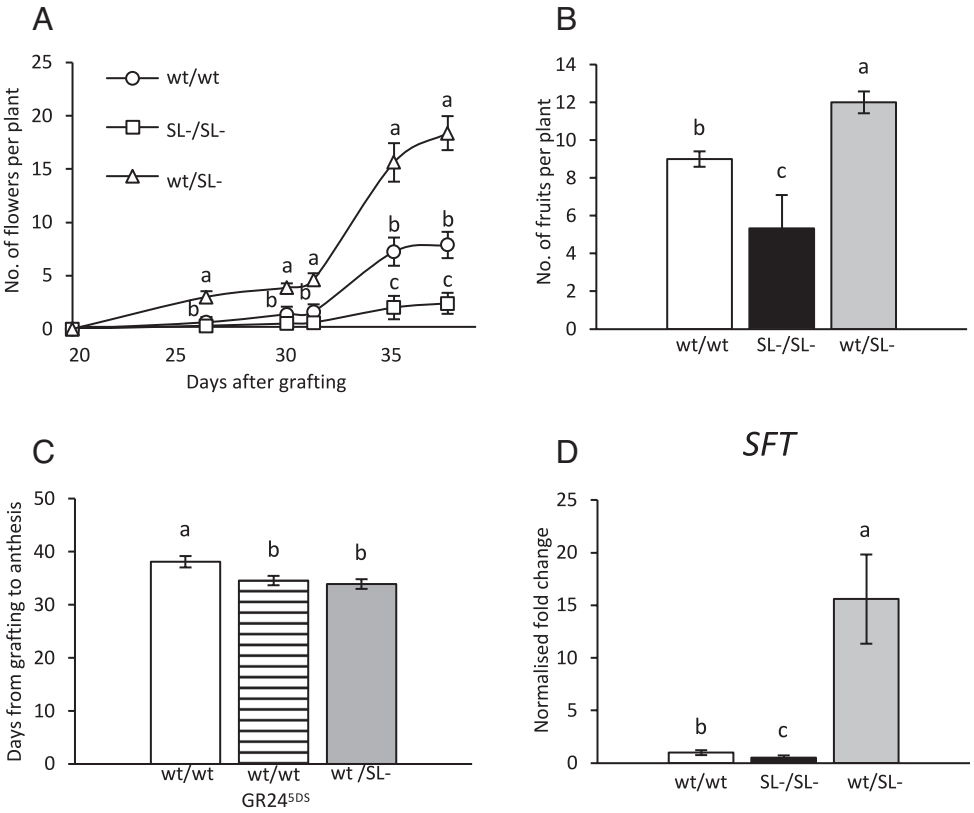

Fig. 1. Effects of different grafting combinations on flowering. (*A*) Number of new individual flowers at anthesis per plant, counted from 20 to 40 d after homo- or heterografting of wt and strigolactone-depleted (SL-) scions and rootstocks. (*B*) Number of ripening fruits 60 d after grafting (and no previous harvest). (*C*) Number of days from grafting to anthesis in homografted wt plants, treated or untreated with 5 μM $GR24^{5DS}$ (25 d after grafting), and heterografted plants. (*D*) Transcript quantification of *SFT* in leaves of different grafts. Transcript abundance was normalized to endogenous *EF1α* and *ACT* and presented as fold-change values over mean values of wt/wt plants, which were set to 1. Data in all panels represent the mean ± SE of n = 5 biological replicates but in panel *C*, where n = 10. All analyses were run in technical triplicates. Different letters indicate significant differences as determined by a one-way ANOVA test ($P < 0.05$) and Tukey's HSD post hoc test.

A consistent trend was apparent in fruit production, with treated plants showing a higher number of fruits [assessed as described earlier (35)] at all time points, compared to mock-treated controls, and more clearly in the early time points (Fig. 2*D*). Interestingly for its possible agronomic implications, the increase in the number of fruits corresponded to a higher cumulative yield per plant, statistically significant starting 63 d after treatment (Fig. 2*E*). All these data suggest that treatment with the synthetic strigolactone analogue $GR24^{5DS}$ promotes flower development by inducing *SFT* expression in reproductive tomato plants, while the effects on floral transition, although present, may be more modest.

Strigolactone-depleted Plants Show an Altered Expression Pattern in the Flowering-related Gene Ontology (GO) Terms. To get an overview of the regulation of the main metabolic processes and signaling pathways overrepresented in the two genotypes, we RNA-sequenced the leaves of 3-wk-old, ungrafted wt and SL- plants; main results are summarized here, while a broader overview can be found in *SI Appendix*, Results. The differentially expressed genes (DEGs) were subjected to GO enrichment analysis, which highlighted over 500 enriched GO terms in the Biological process subcategory (Dataset S1); they were grouped in 40 functional categories that display a very different proportion of up- and down-regulated genes. As shown in *SI Appendix*, Fig. S4, most of the genes related to Developmental processes (GO:0032502) were found to be down-regulated in the SL- plants (191 vs. 64 up-regulated), especially for genes related to Reproduction (GO: 0000003) (114 vs. 34) (Dataset S2). *SI Appendix*, Table S1 shows a list of DEGs linked to Photoperiodism (GO:0048573); Flowering (GO:2000028); Regulation of flower development (GO:0009909); Floral meristem determinacy (GO:0010582); Vegetative-to-reproductive phase transition of meristem (GO:0010228); and Floral organ morphogenesis (GO:0048444). It is important to highlight here that the list includes, among down-regulated DEGs in SL- plants, some well-characterized flowering-related genes such as the floral inducer *SFT*, *FT*-like genes such as *SELF PRUNING 6A* (*SP6A*), and *SBP3*, whose expression is crucial to control the early stages of flower development (36). In addition, we recorded a slight but significant upregulation of *LA* [$\log_2$ fold change ($\log_2$FC) = 0.85] (10, 19). The list also comprises several up- and down-regulated genes related to hormone signaling and biosynthesis, including auxin, gibberellins, ethylene, and brassinosteroids (for a review of their functions, see ref. 37).

Strigolactones Promote Flowering via the miR319-*LA*-*SFT* Module. Both *LA* and the gibberellin biosynthetic and catabolic genes known to be targeted by LA (19) are among the identified DEGs (*SI Appendix*, Results). To investigate the possibility that strigolactones affect them and flowering via miR319, we quantified the mature miR319 form along with *LA* and *SFT* transcripts after spraying wt leaves with $GR24^{5DS}$, and found a rather early induction of mature miR319 followed by the repression of *LA* and the induction of *SFT* (Fig. 3*A*). We also verified that endogenous strigolactones correlate with module activity by quantifying mature miR319 and *LA* transcripts in the leaves of the wt/wt, SL-/SL- and wt/SL- grafted lines (Fig. 3*B*). Thus, besides confirming the

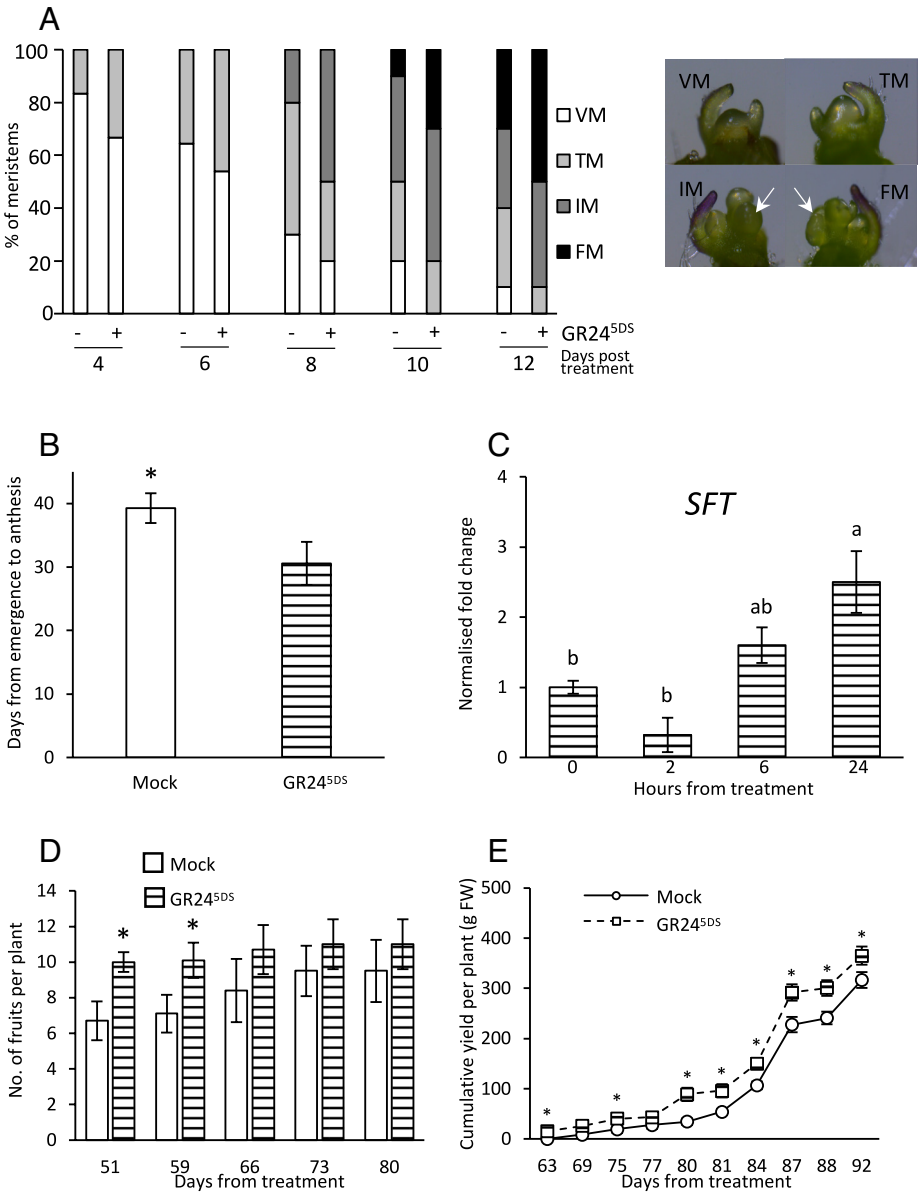

Fig. 2. Effects of GR24^{5DS} treatment on flowering and *SFT* transcription. (*A*) Meristem maturation of mock- or GR24^{5DS} -treated plants. *Right* panel: representative images of the four sequential developmental stages: vegetative meristem (VM), transition meristem (TM), inflorescence meristem (IM), and floral meristem (FM). Plants were treated with a 5 µM solution 4 and 10 d after seedling emergence, i.e., before floral transition and when about 50% of them were at transition. The meristems were evaluated under the stereomicroscope 4 to 12 d after the first treatment (n = 6 to 13). (*B*) Comparisons between mock-treated plants and plants treated with 5 µM GR24^{5DS} 3 wk after seedling emergence, for the number of days from emergence to anthesis. (*C*) Quantification of *SFT* transcript in wt leaves after mock treatment or 2, 6, and 24 h after treatment with 5 µM GR24^{5DS}. Transcript abundances were normalized to endogenous *EF1α* and *ACT* and presented as fold-change values over mean values of mock-treated plants, which were set to 1. Data represent the mean ± SE of n = 5 biological replicates, each analyzed in technical triplicates. Different letters indicate significant differences as determined by a one-way ANOVA test and Tukey's HSD post hoc test ($P < 0.05$). (*D*) Comparisons between mock-treated plants and plants treated with 5 µM GR24^{5DS} 4 wk after seedling emergence, for the number of fruits counted 51 to 80 d from the treatment, and (*E*) average cumulative yield per plant assessed from 63 to 92 d after the treatment. For all tests in *B*, *D*, and *E*, the data represent the mean ± SE of n = 8 biological replicates, and * indicates significant differences between treated and untreated plants for any given time point, as determined by Student's *t* test ($P < 0.05$).

divergent levels of miR319 and *LA* transcripts, our results reveal a positive correlation between strigolactone levels and miR319, also reflected by *LA* (Fig. 3 *A* and *B*) and *SFT* transcript abundance (Figs. 1*D*, 2*C*, and 3*A*).

To obtain a causative link between the promotion of flowering by strigolactones and the miR319-LA-SFT module, we treated with GR24^{5DS} tomato plants that express the *La-2* mutant allele (insensitive to miR319-mediated degradation), under the control of the endogenous *LA* promoter (LA_{pro} >> LA^{m}-*GFP*) (18). The experiment was conducted before floral transition (on 8-d-old seedlings) and confirmed in the first place that strigolactone treatment shortens the time to anthesis (visible in GR24^{5DS}-treated vs. mock-treated wt plants in Figs. 1*C* and 3*C*), but seems not to significantly change the number of leaves at the time of anthesis (Fig. 3*D*), consistently with the observations in *SI Appendix*, Fig. S2 *B* and *C*. Most importantly, no effect of GR24^{5DS} treatment could be detected on LA_{pro} >> LA^{m}-*GFP* plants, demonstrating that a miR319-dependent degradation of *LA* transcripts is necessary for the shortening of flowering time by GR24^{5DS} to occur. Consistently, transcript quantification in leaves treated with GR24^{5DS} shows that strigolactone-induced *SFT* activation is completely dependent on the lowering of *LA* transcripts by miR319 action (Fig. 3*E*). Such effect is visible in the leaves of both vegetative and reproductive plants, but is more marked in the latter.

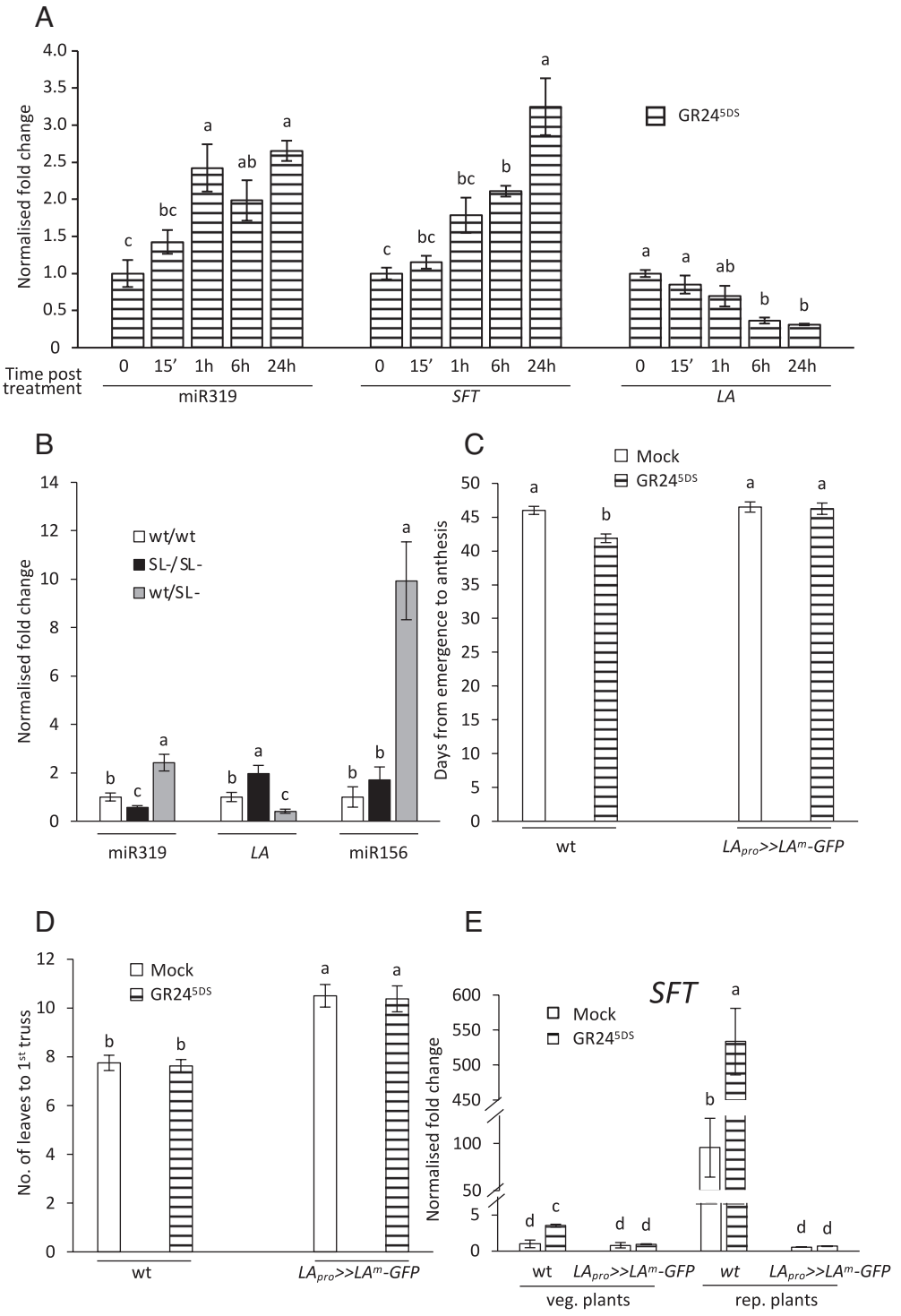

Fig. 3. Strigolactones promote flowering via the miR319-*LA*-*SFT* module. (*A*) treatment with GR24^{5DS} 5 μM rapidly induces the accumulation of mature miR319 and *SFT* transcripts in leaves of 4-wk-old wt plants. (*B*) Effects of endogenous strigolactones on *LA* transcripts and mature miR319 and miR156 quantified in leaves of the graft combinations wt/wt, strigolactone-depleted SL-/SL- and wt/SL- (heterografted plants: wt scions on SL- rootstocks), 2 wk after grafting. (*C*) Exogenous strigolactones must be able to lower *LA* transcripts by increasing miR319 levels to shorten the time to anthesis in tomato. GR24^{5DS} 5 μM was sprayed before floral transition (8 d after seedling emergence) on the leaves of M82 plants (wt) or same-age plants expressing the miR319-resistant *La-2* allele under the control of its own promoter in the same genetic background (LA_{pro} >> LA^m-*GFP*), with n = 8. (*D*) In the same experiment as in *C*, the number of leaves at anthesis was counted. (*E*) Exogenous strigolactones induce *SFT* transcription only if *LA* transcripts are free to decrease in dependence of miR319 increase. *SFT* transcripts were quantified 24 h after treatment with GR24^{5DS} 5 μM on 8-d-old (vegetative) or 4-wk-old (reproductive) plants. In *A*, *B* and *E*, data represent the mean ± SE of n = 5 biological replicates analyzed in technical triplicates. In all panels, letters indicate significant differences as determined by a one-way ANOVA test and Tukey's HSD post hoc test ($P < 0.05$). *SFT* and *LA*-transcript abundances were normalized to endogenous *EF1α* and *ACT*, while mature miR319 and miR156 levels were normalized to *EF1α* and *snRU6* and presented as fold-change values over mean values of untreated wt or wt/wt plants, which were set to 1.

The Effects of Exogenous Strigolactones Can Be Seen Also in Meristems. To assess whether strigolactones may not only affect the flowering network in the leaves, we also quantified (in the meristems, and after treatment with GR24^{5DS}) the transcripts of several genes related to meristem transition and development (38, 39).The experiment was performed on plants treated 1 wk before sampling, either before floral transition (vegetative plants, sampled 15 d after germination) or after (reproductive plants,

sampled at 30 d). It showed that several of them, namely *LA*, *SBP3* and *SBP15*, *FRUITFULL-like1* (*FUL1*) (40), *UNIFLORA* (*UF*) (41), *APETALA1/MICROCALYX* (*AP1/MC*) (42), and *DNA-binding with one zinc finger9* (*DOF9*) (43), are affected not only by age but also by exogenous strigolactones (Fig. 4). This effect attained significant levels especially for treatment after floral transition, even though a nonsignificant trend was often visible also in plants treated before transition. Other genes involved in flower development were also tested, showing a similar trend although not reaching the threshold for statistical significance (*SI Appendix*, Fig. S5). One possible explanation for the lack of significance on genes in *SI Appendix*, Fig. S5 may be, that their window of regulation by $GR24^{5DS}$ may be shifted with respect to the sampling. Alternatively, or additionally—especially for the genes showing a more marked trend (*FA*, *AN*, *DST,* for example)—the possibility exists that the statistical power of our set-up was not sufficient to catch a real difference, or even that strigolactones may impact floral differentiation via a pathway independent of these regulators. Notably, despite the expected auxin-dependent signature in the transcriptome comparison between wt vs SL- leaves (*SI Appendix*, Table S2), the *AUXIN RESPONSE FACTOR5* (*ARF5*) transcripts were not induced in meristems by $GR24^{5DS}$ treatment (*SI Appendix*, Fig. S5). As a whole, this dataset confirms and reinforces the hypothesis that strigolactones affect flowering by promoting meristem maturation and especially flower development.

Strigolactones May Promote Flowering Also by Mitigating Inhibition by Gibberellins. The integrated activities of two core molecular modules, miR156-SBPs and miR319-LA, and of the phytohormone gibberellins act in concert to modulate the transcription of *SFT* in tomato (10). In a previous work, we demonstrated that mature miR156 levels correlate positively with strigolactones, as defective strigolactone biosynthesis prevents drought-triggered miR156 accumulation in leaves, and the synthetic strigolactone analogue $GR24^{5DS}$ induces miR156 (22). As a further confirmation, the heterografted plants (wt/SL-) of this work, in which we see an activation of the strigolactone biosynthetic pathway in leaves (32) and early and profuse flowering along with *SFT* induction (Figs. 1 and 2*C*), also show a marked increase in miR156 levels (Fig. 3*B*). Thus, we reasoned that strigolactones are unlikely to promote flowering by activating the age-related pathway to flowering, in which miR156 should rather decrease to allow *SFT* induction in leaves and the transition from the vegetative to the reproductive phase, as well as flower development. Rather, strigolactones appear to act despite the positive correlation with mature miR156 in leaf cells.

Therefore, we focused on the possible role played by alternative components of the flowering network as mediators of strigolactone effects on *SFT* transcription. Gibberellins were assessed, also considering the proven connection between the miR319-LA module and their biosynthesis (10, 19). The KEGG pathway enrichment analysis of 7140 DEGs between wt and SL- plants confirms widespread dysregulation of genes involved in the biosynthesis of secondary metabolites and signal transduction pathways of plant hormones (*SI Appendix*, Fig. S6). To better understand the role of gibberellins in strigolactone-mediated flowering promotion, we checked the expression of key components of their signaling and biosynthetic pathways (*SI Appendix*, Table S3). We found a downregulation of core signal transduction genes, including the ones encoding the receptors GA-INSENSITIVE DWARF (GID) 1a and GID1b1, the F-box protein SLEEPY1 (SLY1) and the downstream transcription factor PHYTOCHROME INTERACTING FACTOR3 (PIF3) (*SI Appendix*, Table S3). In addition, several genes for biosynthetic enzymes were found differentially expressed between the two genotypes. Those coding for the enzymes that catalyze the last biosynthetic steps toward bioactive gibberellin forms (*SI Appendix*, Fig. S7) were found strongly up-regulated in the SL- line: *Le3OH-23b-hydroxylase* (*GA3ox-2*) and *GIBBERELLIN 20 oxidase-2* (*GA20ox-2*). Instead, *GIBBERELLIN 2 oxidase* (*GA2ox*) genes, encoding enzymes that

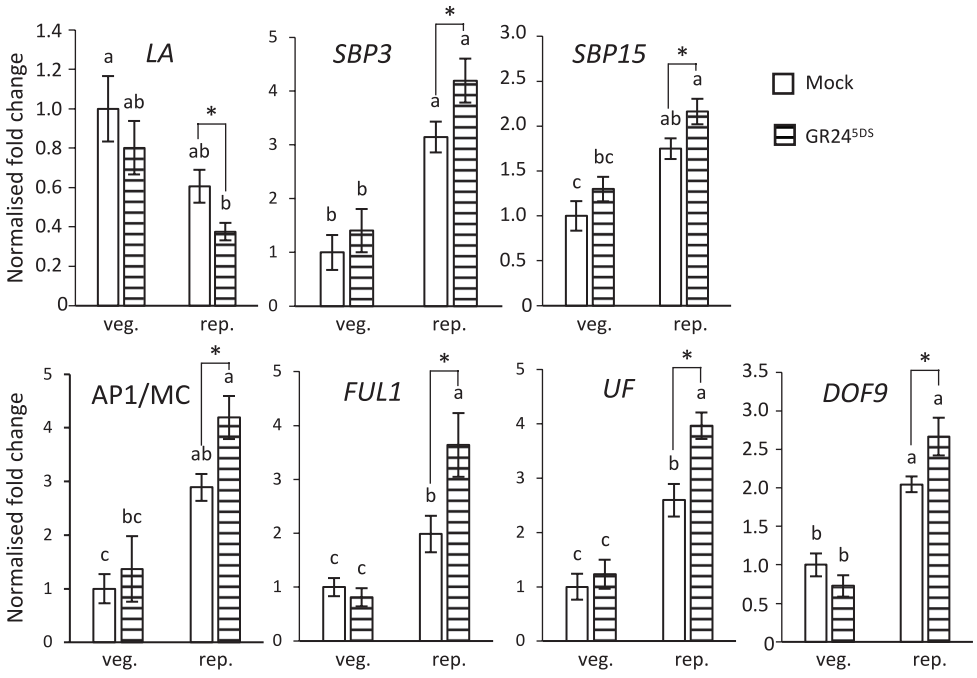

Fig. 4. Effects of exogenous strigolactones on transcripts of marker genes for meristematic development. Vegetative wt plants were treated 8 d after seedling emergence with 5 μM $GR24^{5DS}$ and harvested 1 wk later (veg.); another subset was treated also in the reproductive phase, 23 d after germination, and harvested 30 d after germination (rep.). Transcript abundances were normalized to endogenous *EF1α* and *ACT* and presented as fold-change value over mean values of meristems in untreated vegetative plants, which were set to 1. Data represent the mean ± SE of n = 6 biological replicates (each the pool of 10 apical meristems), analyzed in technical triplicates. Different letters on top of bars indicate statistically significant differences among all samples as determined with one-way ANOVA followed by Tukey's post hoc test; asterisks indicate significant differences for pairwise comparisons between treated and untreated samples of the same age, as detected by Student's *t* test ($P < 0.05$).

lead to inactive gibberellin forms, were either up- (*GA2ox2*, *GA2ox3*) or down-regulated (*GA2ox4*) (*SI Appendix*, Table S3). These results were confirmed via quantitative RT-PCR (qRT-PCR) on independent samples for *GA2ox4*, *GA20ox2,* and *GA3ox2* (*SI Appendix*, Fig. S8). Thus, on balance, the results suggested that more abundant bioactive gibberellins may contribute to the late and reduced flowering in the SL- plants.

To test this hypothesis, we quantified gibberellins in the leaves of the wt and SL- lines. Fig. 5*A* shows a trend toward higher amounts of the bioactive forms GA_1, GA_3, and GA_4 in the latter genotype, which is significant for GA_4. Such metabolites are produced by the sequential action of the GA20- and GA3-oxidase enzymes, the transcripts of which are strongly up-regulated in these plants. The amounts of other biosynthetic gibberellin intermediates and catabolites (*SI Appendix*, Figs. S7 and S9) together with the transcription profile of biosynthetic/catabolic genes (*SI Appendix*, Table S3 and Fig. S8) suggest that gibberellin metabolism is steadily skewed toward more active and less inactive metabolites when strigolactone levels are decreased. Instead, despite the downregulation of genes coding for gibberellin receptors, sensitivity to exogenous gibberellins seemed unaffected in SL- plants, at least in terms of elongation of the first internode upon gibberellin treatment (Fig. 5*B*).

Discussion

Strigolactones Promote Flowering in Tomato. The reproductive defects of strigolactone mutants have been reported anecdotally, without detailed analysis of the possible underlying mechanisms (24–27). Our results show that in tomato, the numbers of flowers and fruits are strictly linked over time to the levels of strigolactones, be they endogenous or exogenous. Furthermore, strigolactones correlate inversely with the time from germination to anthesis. Thus, they offer a promising, innovative research avenue to manage fruiting time and total yield, two commercially pivotal parameters in tomato cultivation.

It is noteworthy that a defect in reproduction has been shown, besides this work, in strigolactone-related mutants of solanaceous and in one legume species, but not in Arabidopsis, rice or pea, despite the early availability of similar mutants in these species. In rice, it has been even shown that a partial loss-of-function of the *CCD7* orthologue increases yield by increasing tillering (44), as does strigolactone insensitivity in *Brassica napus* (45). This suggests that reproduction is affected species-specifically by strigolactones, and that their action superimposes on the conserved pathways controlling flowering, which may be differently wired to each other in different species. In tomato, an anticipated and more profuse flowering induced by strigolactones may well integrate with lower resource allocation to lateral buds (the first hormonal function assigned to strigolactones), namely in genotypes such as the determinate M82 cultivar where overall vegetative growth is limited (2). Also, given the induction of the strigolactone biosynthetic pathway in leaves under drought (32), the hypothesis that strigolactones may contribute to the drought escape mechanism, whereby flowering is brought forward by a previous stress, is worth further investigation.

Strigolactones Affect the Expression of a Large Number of Flowering-related Loci in Leaves and Meristems. The GO enrichment analysis of DEGs obtained from mRNA sequencing of wt and SL- tomato leaves confirmed that, within a wide transcriptional reorganization, the expression of several genes related to the term

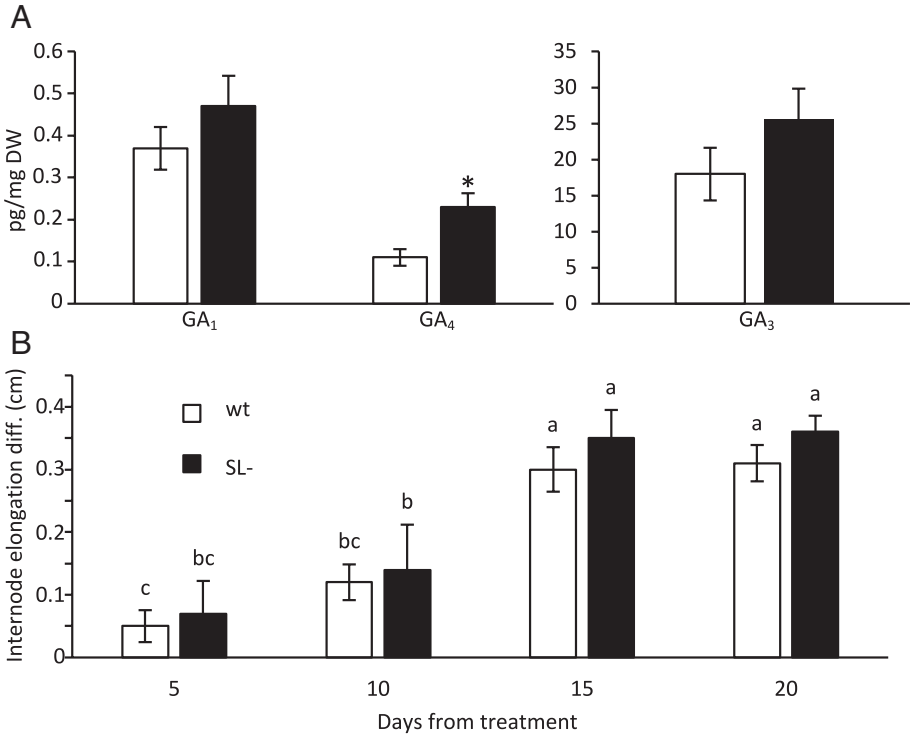

Fig. 5. Effect of strigolactone depletion on gibberellin metabolism and sensitivity. (*A*) Concentration of the active gibberellins GA_1, GA_4 and GA_3 in wt and strigolactone-depleted (SL-) plants, 4 wk after seedling emergence. Data represent the mean ± SE of n = 3 biological replicates analyzed in technical quadruplicates. * indicates significant differences between treated and untreated plants for any given time point, as determined by Student's *t* test ($P < 0.05$). See *SI Appendix*, Fig. S7 for metabolite positioning in the gibberellin pathway. (*B*) Gibberellin treatment (10 μM GA_3) 2 wk after seedling emergence has no different effect on the length increment of the first internode in wt vs. SL- plants, according to pairwise comparison with a Student's *t* test ($P < 0.05$). Data are the difference between the values of the GA_3-treated and mock-treated plants of the same genotype at different time points after treatment and represent the mean ± SE of n = 8 biological replicates. Different letters on top of bars indicate statistically significant differences among all samples as determined with one-way ANOVA followed by Tukey's post hoc test.

Reproduction (GO: 0000003) was altered. Perhaps most notably, this occurred for some crucial flowering genes of the *SP* family. SP factors belong to the CETS (CENTRORADIALIS/TERMINAL FLOWER 1/SP) family, which is shared by all land plants and has been further described in tomato to contain six FT-like proteins (36). Among them, functional analysis confirmed that SP3D/SFT is a major flowering activator that exhibits the same expression in long-day and short-day conditions, and is regulated by the paralogous factors SP5G, SP5G1, SP5G2, and SP5G3 (46, 47). These are flowering repressors with different photoperiodic expression, which are proposed to act via competition with SP3D/SFT for binding in the same functional complex, or for the formation of two different complexes competing for a common target (46). In our analysis, the significant transcript drops for *SP3D/SFT* can alone justify the flowering defects of SL- plants, which are similar to what observed in the *sp3d* mutants (36). The transcriptional decrease of *SP5G*, instead, might be seen as part of an attempted compensation mechanism. Moreover, the zinc-finger transcription factors CONSTANS3 (CO3), and CO-like4a (COL4a) were recently proposed as potential activators of *SFT* in tomato (48), and we found the corresponding genes to be significantly down-regulated in SL- plants (*SI Appendix*, Table S1). Consistently, also genes acting downstream of *SFT* are detectable among our DEGs: The products of *FUL2* and *MADS BOX PROTEIN20* (*MBP20*), which are strongly down-regulated in SL- plants, promote flowering probably by interacting with SFT and SBP factors (*SI Appendix*, Table S1) (40, 49). A peak in the expression of these genes has been detected in the meristem during the vegetative-to-reproductive transition, and is thought to induce tomato flowering additively and to repress inflorescence branching together with *FUL1* (also down-regulated in SL- leaves, *SI Appendix*, Fig. S8, and up-regulated in $GR24^{5DS}$-treated meristems, Fig. 4). In addition, Jointless (J) contributes to maintaining the inflorescence meristem identity and to preventing both the return to a vegetative state and an early conversion to a floral meristem (49). The phenotype observed in the *j* mutants resembles the one seen in the SL- plants (in which *J* is down-regulated, *SI Appendix*, Table S1), at least in terms of delayed flowering. All these DEGs, with others included in Fig. 4 and *SI Appendix*, Table S1 and Figs. S5 and S8 confirm a role for strigolactones in the flowering process. In fact, all genes in these figures, except *DOF9*, are positive regulators of reproduction; the induction of the latter may be seen as an attempt to compensate for the shift toward transition and faster flower development triggered by $GR24^{5DS}$. It should be added that even if much of the supporting transcriptional analysis was done in leaves, leaf transcriptomes are indeed very relevant to floral transition and the speed of flower development because they are the organs that generate the reproductive signal. Thus, while gene activities in meristems are mostly inferred in this work, they are also consistent with phenotypes and are indeed validated in meristems, in some specific examples.

Positioning Strigolactones in the Flowering Network of Tomato: A Connection with the miR319-LA Module and Gibberellins. Looking to define a molecular link between strigolactones and *SFT* expression, we investigated the three main flowering pathways described in tomato: the age-, the gibberellin-, and the miR319-LA dependent (10).

Considering the positive correlation between strigolactones and mature miR156 levels (22) (this work, Fig. 3*B*), the age pathway was deprioritized, while we investigated more in depth the gibberellin pathway by RNAseq, metabolite analysis, and sensitivity assays. In our DEGs set, transcripts of the biosynthetic genes *GA3ox-2* and *GA20ox-2* (50) were much more concentrated in SL- leaves than in the wt. On the other hand, expression of catabolic *GA2ox* genes (50) followed divergent patterns: *GA2ox4* was found to be down-regulated, which would rather push for more bioactive gibberellins. Conversely, the upregulation of *GA2ox2* and *GA2ox3* in the transgenic line could be seen as an attempt to keep gibberellin homeostasis (*SI Appendix*, Table S3). The expression changes in gibberellin biosynthetic and catabolic genes are confirmed by hormone quantification (Fig. 5*A*); in fact, the concentrations of bioactive gibberellins and of their intermediates tended to be higher in SL- plants, while lower for the inactive catabolites. This trend indicates that strigolactones may indeed mitigate gibberellin effects on flowering in tomato by decreasing their biosynthesis without affecting perception (as suggested by the internode elongation test, Fig. 5*B*). It is tempting to speculate here that the lack of reproductive defects in strigolactone-related mutants of Arabidopsis and other model species may be due to the opposite effect of gibberellins on flowering, as in tomato vs. Arabidopsis (10, 14). In regard to the strigolactone-gibberellin connection, it is also worth noting first that a reverse relationship—gibberellin inhibiting the biosynthesis of strigolactones—has been reported in rice (51). Second, previous work has described the strigolactone-dependent physical interaction between the strigolactone receptor DWARF14 (D14) and the DELLA protein in rice (52). Although later considered not relevant in the context of branching control, it may be worth exploring whether the interaction with D14 is conserved for the only tomato DELLA protein PROCERA and whether it may rather be relevant for flowering. Indeed, the effects of DELLAs in vegetative and reproductive development are genetically separable (17).

The miR319-LA module is the third flowering pathway characterized in tomato (10); we confirmed here its role, and the divergent profile of mature miR319 and of the *LA* and *SFT* transcripts. We also added a tight link to strigolactones. In fact, we found significantly more mature miR319 in the wt in comparison to the SL- plants. Moreover, its levels were even higher in leaves treated with $GR24^{5DS}$ and in the leaves of wt/SL- plants, where the strigolactone-biosynthetic pathway is overactivated. Finally, we could establish a definitive cause-effect link between the promotion of flowering by exogenous strigolactones, the activation of *SFT* and the degradation of *LA* transcripts by miR319. In fact, no induction of *SFT* transcripts by $GR24^{5DS}$ treatment could be observed in vegetative or reproductive tomato plants expressing a miR319-resitant version of *LA*. $GR24^{5DS}$ treatment accelerated meristem maturation, although it did not significantly affect the number of leaves at anthesis. This apparent discrepancy with the phenotype of wt/SL- plants, for which the number of leaves at anthesis was reduced instead, may be due to the persistent action of slightly overactivated synthesis in heterografted plants vs a pulse treatment with $GR24^{5DS}$, and to the inherently lower power of a statistical test on the number of leaves vs the number of days to anthesis. Importantly, earlier anthesis associated with high strigolactone levels is likely due to a promotion of flower development via the miR319-LA-SFT module. This is consistent with the known role of SFT on flower development (6).

Finally, it is worth noting again here that LA has been characterized not only as a direct repressor of flowering genes, *SFT* included, but also as an inducer of the gibberellin pathway. In fact, miR319 overexpression in tomato leads to lower gibberellin content via downregulation of *GA20ox1* and upregulation of *GA2ox4*. The opposite happens in plants not expressing miR319 or expressing a miR319-resistant form of *LA* (19), thus coming full circle with the strigolactone-dependent increase of bioactive gibberellins. In our dataset, *GA20ox2* (a close paralogue of *GA20ox1*) is indeed strongly up-regulated in SL- plants, while *GA2ox4* is down-regulated; and bioactive gibberellins are higher

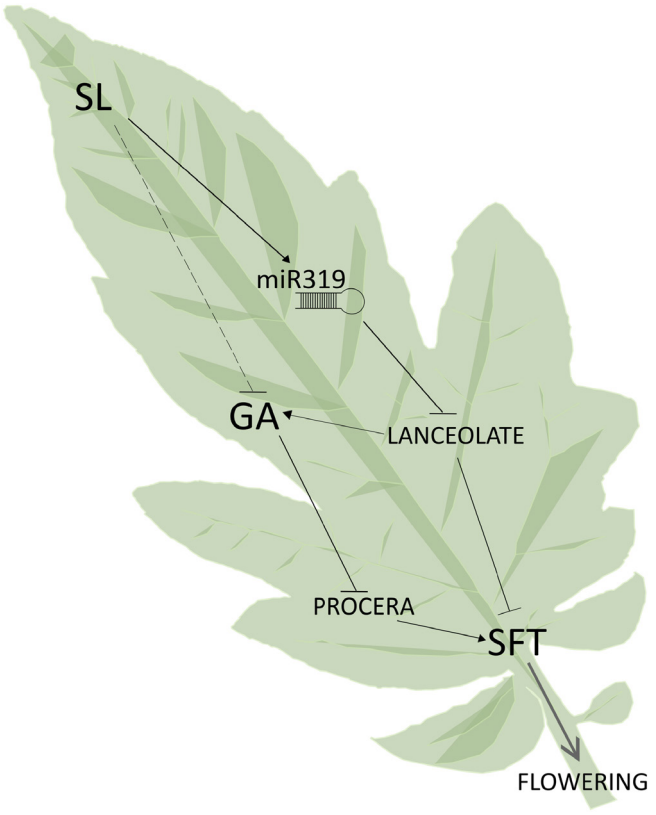

Fig. 6. Role of strigolactones in SINGLE FLOWER TRUSS (SFT) induction in leaves. Strigolactones (SL) induce the accumulation of mature miR319, leading to a drop in the concentration of *LANCEOLATE* transcripts and thus, to an increase in *SFT* expression. The *LANCEOLATE* decrease would also mitigate the gibberellin (GA) pathway and enhance the activity of the only DELLA protein of tomato, PROCERA (10). Whether the effect of strigolactones on gibberellin content may also partly be independent of *LANCEOLATE* is still to be assessed.

(*SI Appendix*, Table S3 and Fig. 5*A*). Note that in spite of the known role of auxins in reproduction and effects of strigolactones on auxin fluxes (53), and the fact that the expected signature of altered auxin signaling was detected in our leaf transcriptome of SL- plants (*SI Appendix*, Table S2), the auxin-dependent factor *ARF5* (homolog of Arabidopsis *MONOPTEROS*), which is important for reproduction in tomato (53) was not significantly induced by $GR24^{5DS}$ in meristems (*SI Appendix*, Fig. S5). This is consistent with the fact that similar auxin concentrations were found in flowers of wt and strigolactone-depleted Lotus plants (24). On the other hand, the auxin-dependent repressor of flowering *DOF9* is induced by treatment in meristems (Fig. 4), and *ARF3* (homolog of Arabidopsis *ETTIN*) (53) is down-regulated in SL- leaves (*SI Appendix*, Table S2). Thus, more investigations are necessary to rule out or confirm the possible contribution by auxin to strigolactone-dependent reproductive defects. Fig. 6 summarizes our findings and proposes a draft model of strigolactone interactions within the flowering network in tomato leaves. Future work aimed at describing the transcriptome in meristems of wt vs. SL- vs. heterografted SL-/wt plants, along with full phytohormonal profiling, will help refine the findings in our study and add components and connections to this sketch.

Conclusions

This study aimed to establish the effect of strigolactones on flowering in tomato and justify the reproductive phenotype of strigolactone-related mutants in this species. We show that strigolactones accelerate floral transition to a certain extent, and especially flower development; and that their levels correlate with the number of flowers and fruits in tomato. Furthermore, we demonstrate that impaired strigolactone synthesis causes a dysregulation of several pathways involved in flowering and propose the miR319-LA module as a key link between strigolactones, *SFT* transcription, and gibberellin content in leaves. Our study positions strigolactones in the flowering regulation network of a model crop species and opens to applicative impacts in the management of tomato fruiting time and total yield.

Materials and Methods

Details on the materials and methods used in our manuscript are provided in *SI Appendix, Materials and Methods* on the PNAS website, including on:

Plant Material, Observations, and Treatments. The tomato *SlCCD7*-silenced line 6936 and its wt genotype M82 were a kind gift by H. J. Klee (University of Florida) (28); the LA_{pro} >> La^{m}-*GFP* genotype was published earlier (18) along with procedures for meristem observations and determination of floral transition. Grafting and treatment with $GR24^{5DS}$ were performed as reported (32).

Molecular Procedures. Library construction, sequencing and processing of mRNA data, functional analysis of tomato DEGs, gene transcript quantification by qRT-PCR, gibberellin quantification by ultrahigh performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS), and statistical analysis were conducted according to established procedures for which details are published as *SI Appendix*.

Data, Materials, and Software Availability. Raw sequencing data can be found at the Gene Expression Omnibus (GEO) under the series record GSE264066 (accession numbers GSM8209523, GSM8209524 and GSM8209525 for the wt genotype; WWGSM8209505, GSM8209506 and GSM8209507 for the SL- plants) (54).

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