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Inhibition of flowering by gibberellins in the woody plant *Jatropha curcas* is restored by overexpression of *JcFT*

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ABSTRACT

Jatropha curcas (*J. curcas*) is a perennial oil-seed plant with vigorous vegetative growth but relatively poor reproductive growth and low seed yield. Gibberellins (GAs) promotes flowering in most annual plants but inhibits flowering in many woody plants, including *J. curcas*. However, the underlying mechanisms of GA inhibits flowering in perennial woody plants remain unclear. Here, we found that overexpression of the GA biosynthesis gene *JcGA20ox1* inhibits flowering in *J. curcas* and in *J. curcas* × *J. integerrima* hybrids. Consistent with this finding, overexpression of the GA catabolic gene *JcGA2ox6* promotes flowering in *J. curcas*. qRT—PCR revealed that inhibits floral transition by overexpressing *JcGA20ox1* resulted from a decrease in the expression of *JcFT* and other flowering-related genes, which was restored by overexpressing *JcFT* in *J. curcas*. Overexpression of *JcGA20ox1* or *JcGA2ox6* reduced seed yield, but overexpression of *JcFT* significantly increased seed yield. Furthermore, hybridization experiments showed that the reduction in seed yield caused by overexpression of *JcGA20ox1* or *JcGA2ox6* was partially restored by the overexpression of *JcFT*. In addition, *JcGA20ox1*, *JcGA2ox6* and *JcFT* were also found to be involved in the regulation of seed oil content and endosperm development. In conclusion, our study revealed that the inhibitory effect of GA on flowering is mediated through *JcFT* and demonstrated the effects of *JcGA20ox1*, *JcGA2ox6* and *JcFT* on agronomic traits in *J. curcas*. This study also indicates the potential value of GA metabolism genes and *JcFT* in the breeding of new varieties of woody oil-seed plants.

1. Introduction

In angiosperms, floral transition is a key developmental switch from vegetative to reproductive growth that requires precise regulation to maximize reproductive success. Ongoing investigations on floral transition in *Arabidopsis thaliana* (*Arabidopsis*) and other species have revealed that the photoperiod pathway, vernalization pathway, autonomous pathway, temperature pathway, GA pathway, and age pathway are involved in the regulation of flowering time (Cho et al., 2017; Teotia and Tang, 2015; Freytes et al., 2021). *FLOWERING LOCUS T* (*FT*), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), and *LEAFY* (*LFY*) are key internal flowering transcription factors (Goldberg-Moeller et al., 2013; Li et al., 2014) that integrate signals from the six upstream pathways and then activate the downstream floral organ development genes *APETALA 1* (*AP1*), *AGAMOUS* (*AG*), *SEPALLATA* (*SEP*), and *FRUITFULL* (*FUL*), which determine flower formation (Lee and Lee, 2010; Mouradov et al., 2002; Parcy, 2005).

There are 136 types of GAs with defined structures that have been identified in nature, but among them, only GA_1, GA_3, GA_4, and GA_7 physiologically regulate plant growth (Yamaguchi, 2008). In the GA synthesis and metabolism pathways, GA 20-oxidases (GA20ox) are key enzymes involved in GA biosynthesis, which catalyze the formation of

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active GAs, whereas GA2 oxidases (GA2ox) are the major GA catabolism enzymes, which degrade active GAs to form biologically inactive GAs (Olszewski et al., 2002). GAs play crucial roles in promoting cell division and elongation (Oh et al., 2014), seed dormancy and germination (Resentini et al., 2015; Li et al., 2020), hypocotyl and stem elongation (Park et al., 2003), root development (Yaxley et al., 2001), and flowering (Bao et al., 2020; Wilson et al., 1992). In *Arabidopsis*, the role of GAs in regulating flowering was first elucidated through the application of GAs (Langridge, 1957). GA induces *FT* transcription in *Arabidopsis* leaves to promote flowering under long daylight (LD) conditions (Langridge, 1957; Porri et al., 2012). However, *FT* expression is drastically repressed in the GA-deficient mutant *ga1–3* or in plants with impaired GA signaling (*dellaΔ17*), whereas it is significantly induced in exogenously GA-treated plants or in persistently active GA mutants (Porri et al., 2012; Galvão et al., 2012). *FT,* one of the major members of the phosphatidyl ethanolamine-binding protein family, plays an important role in the integration of the six major flowering pathways and accelerates flowering in *Arabidopsis* under LD conditions (Bao et al., 2020; Hisamatsu and King, 2008). As corepressors of the GA pathway, DELLAs regulate the activity of a number of transcription factors in leaves and shoot tips, thereby regulating flowering. DELLAs largely inhibit *FT* transcription by negatively affecting several *FT*-activating factors, such as CONSTANS (CO). DELLAs bind directly to CO through their CCT domain and sequester CO to the *FT* promoter, thus directly down-regulating *FT* and *TSF* expression at low GA levels (Bao et al., 2020; Wang et al., 2016). Furthermore, by competitively binding to CO, DELLAs may inhibit the interaction between CO and nuclear factor Y (NF-Y) subunit B (NF-YB), thereby suppressing flowering (Zhang et al., 2023). DELLAs may also regulate the expression of *FT* blocking the transcriptional activation of *FT* via the *PHYTOCHROME INTERACTING FACTOR 4* (*PIF4*) in warm environments (Kumar et al., 2012). In addition, the bHLH transcription factor MYC3 interacts with and stabilizes DELLAs, and MYC3 directly represses *FT* expression, resulting in late flowering (Bao et al., 2019). Under short daylight (SD) conditions, the non-flowering phenotype of the *ga1* mutant can be rescued by overexpressing *LFY* or *SOC1* (Moon et al., 2003; Blázquez et al., 1998). Moreover, a constitutively expressed *LFY* transgene was able to restore flowering in GA-deficient *ga1–3* mutants under SD conditions (Blázquez et al., 1998). DELLAs regulate the expression of floral meristem organization genes in complex patterns. Under non-inducible SD conditions, DELLAs inhibit the transcriptional activity of SQUAMOSA-PROMOTER BINDING PROTEIN-LIKE (SPL), thereby repressing the expression of *SOC1* and *FUL* (Dong et al., 2017). SPL9 recruits DELLA proteins to the *AP1* motif and subsequently induces *AP1* expression to promote the transformation of lateral primordia into flowers (Yu et al., 2012).

In perennials, GA application inhibits flowering in avocado (Salazar-García and Lovatt, 1998), *Citrus sinensis* (Muñoz-Fambuena et al., 2012), and sweet cherry (Lenahan et al., 2006), whereas the GA biosynthesis inhibitor Paclobutrazol (PAC) enhances reproductive development in mango (Winston, 1992) and *Litchi chinensis* (Menzel and Simpson, 1990). In grapevine, it was reported that tendrils were converted to inflorescences in a dwarf GA-insensitive grapevine mutant (Boss and Thomas, 2002), indicating that GA inhibits flowering. In *J. curcas*, the application of GA also inhibits flowering, whereas the application of PAC promates flowering (Li et al., 2018; Song et al., 2013). The results of these studies indicated that GA plays different roles in annual and perennial plants, but the molecular mechanisms underlying GA-regulated flowering in perennials are not yet known due to the lack of genetic transformation techniques for most perennials.

Jatropha curcas (*J. curcas*) and *Jatropha integerria* (*J. integerrima*, abbreviated as *Ji*) are perennial woody plants belonging to the Euphorbiaceae family. *J. curcas* has great potential as a renewable energy crop due to the high oil content of its seeds, with a seed oil content of 30–40% and a kernel oil content of approximately 50%. However, the low yield of this plant is an obstacle to its industrialization. The vegetative shoots and leaves of *J. curcas* are overabundancet ; therefore, reduction of unwanted vegetative growth is essential. In addition, another important reason for the low productivity of *J. curcas* seeds is poor flowering (Divakara et al., 2010). Fortunately, several techniques have been introduced to improve seed production in *J. curcas,* including increasing seed yield by shortening flowering time (Ye et al., 2014), increasing the number of female flowers by treatment with cytokines (Pan et al., 2016), and increasing seed size by overexpressing *JcARF19* (Sun et al., 2017).

In this work, we investigated whether GA plays a key role in the regulation of flowering time in *J. curcas* through the application of GA, the overexpression of GA oxidase genes and genetic complementation experiments. Our results show that the *JcGA20ox1* gene inhibits floral transition and the *JcGA2ox6* gene promotes floral transition. Furthermore, overexpression of *JcGA20ox1* reduced the number of inflorescences and florets, whereas overexpression of *JcGA20ox6* did not affect the number of inflorescences but reduced the number of florets. Using qRT—PCR analysis and hybridization experiments, we demonstrated that *JcGA20ox1* play suppressing flowering role through decreased the espression of *JcFT* since the *JcGA20ox1*-suppressed flowering phenotype was restored after the overexpression of *JcFT*. Through hybrid experiments, we also found that *JcGA20ox1* inhibited floral transition in the *J. curcas* × *J. integerrima* hybrid. *JcGA20ox1* decreased not only *JcFT* but also *JiFT* expression, and dual decreased caused a significandelay in the flowering of the *Jc* (*JcGA20ox1*-OE) × *Ji* hybrid plants; the flowering time of the F1 hybrids was significantly later than that of the parental plants. Overexpression of one of the *JcGA20ox1*, *JcGA2ox6*, or *JcFT* genes reduced both the seed and kernel oil content. Overexpression of *JcGA20ox1* or *JcGA2ox6* decreased annual seed yield, whereas *JcFT* overexpression increased annual seed yield, and the *JcFT* gene partially rescued the decrease in annual seed yield caused by overexpression of *JcGA20ox1* or *JcGA2ox6*. Our results suggest that GAs play an important role in the floral transition of *J. curcas* and revealed that the inhibition of floral transition by GAs may occur through *JcFT*, and demonstrating the values of the *JcGA20ox1*, *JcGA2ox6* and *JcFT* genes in *J. curcas* breeding. Taken together, our results provide guidance for the study of floral transition and breeding in *J. curcas* and other woody plants

2. Materials and methods

2.1. Plant materials and growth conditions

Wild-type (WT), transgenic and hybrid plants were grown in the field at the Xishuangbanna Tropical Botanical Garden of the Chinese Academy of Sciences (XTBG, 21°54′N, 101°46′E, 580 m above sea level) in Mengla County, Yunnan Province. The planting density was 3 × 3 m. T1 transgenic seeds of 35S: *JcGA20ox1* (*JcGA20ox1*-OE) L1/L4, *JcUEP:JcGA2ox6* (*JcGA2ox6*-OE) L8/L43 and *AtSUC2*:*JcFT* (*JcFT*-OE) L15 plants were collected from field-grown plants (Li et al., 2014; Ni et al., 2015; Hu et al., 2017). All seeds were germinated and grown in a growth chamber under a 16-h light/8-h dark cycle at 28 °C. Agronomic traits were recorded for 15 or 30 individuals. Field trials were carried out in 2021 and 2022. Letters indicate significant differences between genotypes according to one-way ANOVA with Tukey's HSD post hoc test ($P<0.05$).

2.2. GA_{4+7} and PAC treatment

One gram of GA_{4+7} powder (BBI Life Sciences Corporation, Shanghai, China) or 5 g of 25% PAC powder (BBI Life Sciences Corporation, Shanghai, China) was mixed with 100 kg of soil mixture and added to a large watertight pot. The shelf life of GA_{4+7} and PAC is three and five years respectively. Thirty WT seeds were germinated in the control soil and in the soil mixed with GA_{4+7} and PAC, and 10 of each seedling was used for flowering time analysis.

2.3. qRT–PCR/gene expression analysis

Total RNA was isolated from *J. curcas* tissues as previously described (Ding et al., 2008). Total RNA was isolated from *J. integerrima* tissues using the silica gel method. One microgram of total RNA was reverse transcribed using the PrimeScript™ RT Reagent Kit together with the gDNA Eraser. Quantitative real-time PCR (qRT-PCR) was performed on cDNA using LightCycler® 480 SYBR Green I Master Mix (Takara, Dalian, China). All *J. curcas* gene expression data obtained via qRT—PCR assay were normalized to the reference gene *JcACTIN1*. Since there is no information on the genome of *J. integerrima*, the *JiACTIN1* and *JiFT* fragments of ~1100 bp and ~500 bp, respectively, were amplified using degenerate primers. Since *JcACTIN1* and *JiACTIN1* have high sequence similarity, and the qRT–PCR primers for *JcACTIN1* also amplified *JiACTIN1* fragments, so the same primers were used to amplify the fragments of the internal reference genes (*JcACTIN1* and *JiACTIN1*) in *J. curcas* and *J. integerrima*. The sequences of the primers used for qRT—PCR are listed in Supplementary Table 2. Three independent biological replicates were performed for each sample, and three technical replicates were performed for each biological replicate.

2.4. Hybrid experiments

For controlled hand-pollination experiments on *J. curcas*, mature pollen collected from *JcFT*-OE plants was applied to the female stigmas of *JcGA20ox1*-OE or *JcGA20ox6*-OE plants to obtain hybrid *JcGA20ox1*-OE × *JcFT*-OE (*JcGA20ox1* × *JcFT*) or *JcGA20ox6*-OE × *JcFT*-OE (*JcGA20ox6* × *JcFT*) plants. For the purpose of controlled hand pollination in hybrid experiments between *J. curcas* and *JcGA20ox1*-OE (designated *Jc* (*JcGA20ox1*-OE)), mature pollen collected from wild-type *J. integerrima* was applied to the female stigma of *Jc* (*JcGA20ox1*-OE) plants to obtain the hybrid *Jc* (*JcGA20ox1*-OE) × *Ji*. For the hybridization experiments, flowers were bagged for one week after cross-pollination. After two months, the mature seeds were collected and placed in a greenhouse for germination and growth. One month after germination, DNA was extracted from the young leaves of the seedlings, and after which the hybrid seedlings were identified via PCR using the primers XT126/XB182, XE788/XE789 and XD626/XD627 (Supplementary Table). Fifteen *JcGA20ox1* × *JcFT* and *JcGA0ox6* × *JcFT* hybrid plants and five *Jc* (*JcGA20ox1*-OE) × *Ji* hybrid plants were obtained. All the plants were planted in the field, and statistical analysis and phenotypic observation were subsequently performed.

2.5. Characterization of plant traits

The plant height and number of branches per plant were calculated for seven-month-old plants or one-year-old plants. The number of branches per plant was calculated for one-year-old plants. The flowering time was recorded from germination to bolting. The number of inflorescences was counted according to the total number of inflorescences produced per plant in one year. The ten autumn-ripened fruits and seed sizes at maturity were measured with Vernier callipers. The weights of ten seeds and ten kernels were determined using a precision electronic balance after the seeds and kernels had been placed in an oven at 37 °C for 7 days to remove moisture. The oil content of the dried seeds and kernels was measured using a mq-one-seed and olive analyzer (Bruker Optik GmbH, Ettlingen, Germany). Paraffin sections were cut from the seeds to observe cell morphology using a fluorescence microscope (Leica, Heerbrugg, Switzerland).

2.6. GAs content determination

The GA content was quantified according to a previously described method (Liu et al., 2019). Three replicate stem samples were used. The frozen plant samples were placed in Eppendorf tubes containing 400 pmol/L [$^2H_2$]GAi and ground by hand to a fine powder in liquid nitrogen using a homemade stretched glass rod. Then 100 μl of 30 mmol/L EDC in EtOH solution was added to the tubes. The mixture was left in a constant temperature water bath at 40 °C for 5 hours without shaking for one-pot sample preparation. After the mixture was incubated in a water bath, it was centrifuged at 8000 rpm for 10 minutes, the supernatant was collected, and the residue was washed twice with 50 μl of EtOH. The supernatants were combined and evaporated using a nitrogen evaporator under a gentle nitrogen stream and redissolved in 50 μl H_2O for UHPLC—ESI—MS/MS analysis.

2.7. Statistical analysis

Data analysis was performed using Statistical Products and Services Solution version 21.0 software (SPSS). All P values were determined by one-way ANOVA with Tukey's or Tamhane's post hoc tests. Graphs were generated using the Origin version 2021.

3. Results

3.1. GA represses floral transition in J. curcas

PAC is an inhibitor of GA biosynthesis. To confirm exogenous GA effects on the *J. curcas* floral transition, we grow *J. curcas* plants in soil supplemented with 1 g of GA_{4+7} and 5 g of 25% PAC. We found that the flowering time of plants continuously treated with GA_{4+7} was approximately 60 days later than that of control plants (Fig. 1A-C). Conversely, plants treated with PAC flowered approximately 40 days earlier (Fig. 1A-C). Taken together, these results suggest that treatment with GA_{4+7} inhibits floral transition, whereas treatment with PAC promotes floral transition. In summary, we demonstrated that GA_{4+7} also inhibits the floral transition of *J. curcas*. Additionally, we observed that exogenous GA was involved in the regulation of plant architecture, plants treated with GA_{4+7} were taller and produced more branches than WT plants, while PAC-treated plants were shorter and producing fewer branches (Fig. 1A-C, F).

3.2. Overexpression of JcGA20ox1 represses flowering, while overexpression of JcGA2ox6 promotes flowering in J. curcas

JcGA20ox1 is a member of the *J. curcas JcGA20ox* oxidase gene family, *JcGA2ox6* is a member of the *J. curcas JcGA2ox* oxidase gene family. In our previous studies, transgenic plants overexpressing *JcGA20ox1* (a member of the *J. curcas JcGA20ox* oxidase gene) under the control of the 35S promoter and transgenic plants overexpressing *JcGA2ox6* (a member of the *J. curcas JcGA2ox* oxidase gene) driven by a weak constitutive *JcUEP* promoter was obtained, but the flowering time of these transgenic plants was not analyzed (Ni et al., 2015; Hu et al., 2017). To investigate the role of GAs in the floral transition of *J. curcas*, seeds collected from the T0 generation *JcGA20ox1*-OE L1 and L4 transgenic lines and *JcGA2ox6*-OE L8 and L43 transgenic lines with significant changes in plant height were germinated, and the content of GAs and gene expression levels in 2-month-old T1 generation seedlings was determined. The flowering time of these transgenic progeny plants was recorded in the field.

Our assay showed that overexpression of *JcGA20ox1* increased the levels of biologically active GA_4 (Fig. S1A), whereas overexpression of *JcGA2ox6* decreased the levels of biologically active GA_4 (Fig. S1A, B), indicating that *JcGA20ox1* and *JcGA2ox6* are key biosynthetic and degradative enzymes, respectively. In addition, the expression of *JcGA20ox1* was upregulated approximately 29-fold and 782-fold in the T1 generation *JcGA20ox1*-OE transgenic lines L1 and L4, respectively (Fig. 2B), and approximately 13-fold and 793-fold in the T1 generation *JcGA2ox6*-OE transgenic lines L8 and L43, respectively (Fig. 2C). With respect to flowering time, there was a clear difference between the T1 generation of *JcGA20ox1*-OE and *JcGA2ox6*-OE transgenic plants. Compared to the WT plants, the T1 generation of *JcGA20ox1*-OE

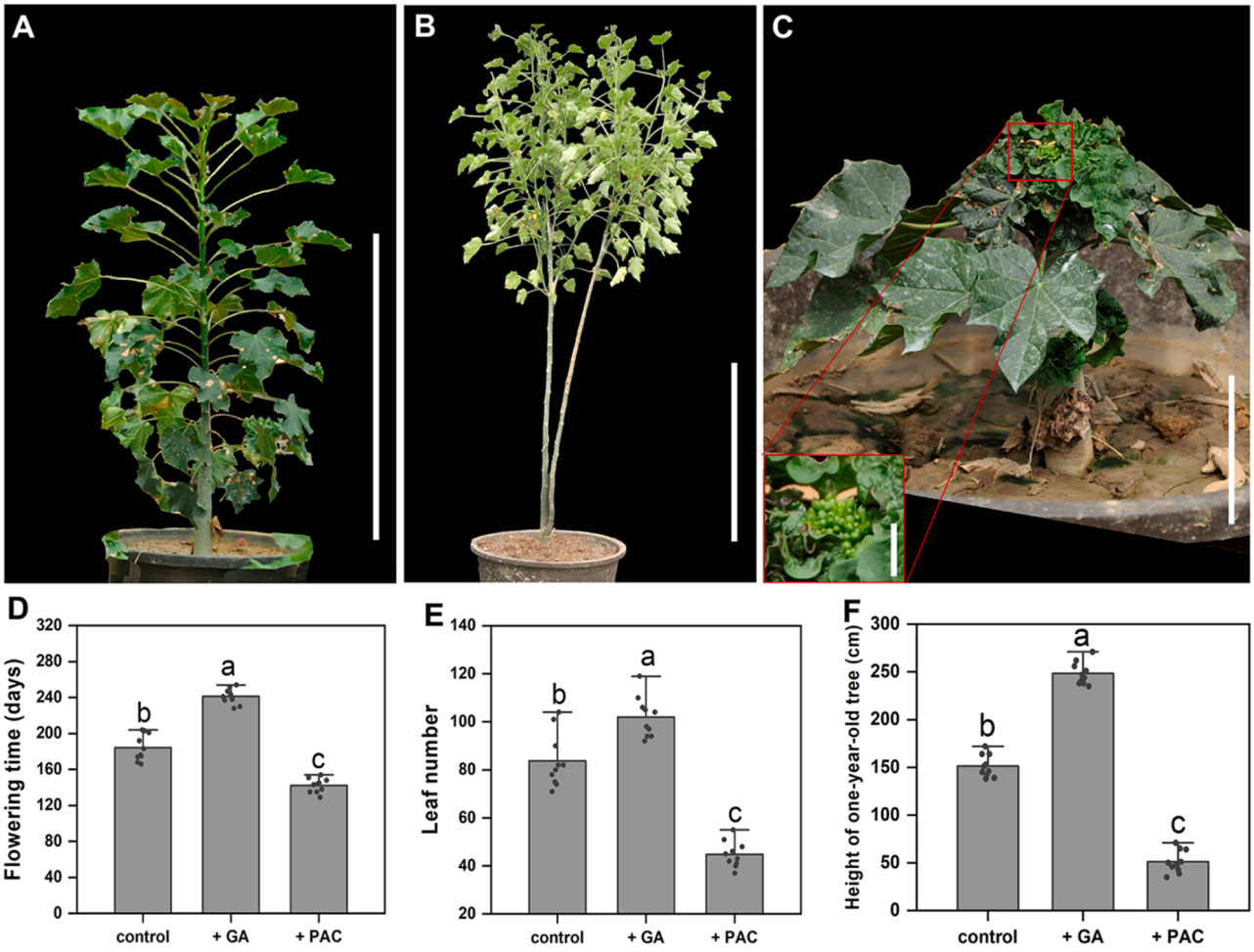

Fig. 1. Gibberellin suppresses floral transition in *J. curcas*. (A) Four-month-old seedlings of control plants were grown in the greenhouse; scale bar = 1 m. (B) Four-month-old plants treated with 1 g of GA_{4+7}. GA_{4+7} powder was added to the soil before planting; bar = 1 m. (C) Four-month-old seedlings were treated with 1 g of 25% PAC. 25% PAC powder was mixed into the soil before planting. The insects provide a close-up view of the inflorescences. Bar = 10 cm for plants. Bar = 1 cm for inflorescences. (D-E) Flowering time and number of leaves on the main stem at flowering. (F) The heights of one-year-old trees were analyzed for the control and for those plants treated with GA (+GA) or PAC (+PAC). For D-F, the data are presented as the mean ± SD (n = 10). Single data points are plotted. Letters indicate significant differences between genotypes according to one-way ANOVA with Tukey's HSD post hoc test ($P<0.05$).

transgenic plants exhibited later flowering, whereas the T1 generation of *JcGA2ox6*-OE transgenic plants exhibited earlier flowering. The flowering time of the *JcGA20ox1*-OE L1 and L4 plants was approximately 60 days later than that of the WT plants, whereas the flowering time of the *JcGA2ox6*-OE L8 and L43 plants was 15 days earlier than that of the WT plants (Fig. 2A, D). These results suggest that overexpression of *JcGA20ox1* prevents flowering and that overexpression of *JcGA2ox6* promotes flowering, which is consistent with the results of GA_{4+7} and PAC treatment (Fig. 1A-C).

The number of inflorescences and florets was also affected by GA in *J. curcas*. We analyzed the inflorescences produced by WT and transgenic plants in the first year and showed that the *JcGA20ox1*-overexpressing plants produced fewer inflorescences, whereas *JcGA2ox6*-overexpressing plants produced inflorescences that to those produced by the WT plants (Fig. 2E); however, we found that the overexpression of either *JcGA20ox1* or *JcGA2ox6* reduced the number of florets in the inflorescences, including both female and male flowers (Fig. 2F, G), but did not affect the ratio of male to female flowers (Fig. 2H).

3.3. The expression levels of flowering-related genes were altered in *JcGA20ox1*-OE and *JcGA2ox6*-OE *J. curcas*

To elucidate the molecular mechanisms underlying the effect of GA on the floral transition in *J. curcas*, we analyzed the expression of genes involved in the GA signaling and flowering pathways in T1 generation *JcGA20ox1*-OE and *JcGA2ox6*-OE transgenic plants. The results showed that overexpression of *JcGA20ox1* increased the expression of the GA receptor *JcGID1* (Fig. 3A), and overexpression of *JcGA2ox6* increased the expression of the DELLA protein *JcGAI* (Fig. 3B). Overexpression of *JcGA20ox1* significantly decreased the expression of the flowering-integrated gene *JcFT,* whereas overexpression of *JcGA2ox6* increased the expression of *JcFT* and *JcSOC1* (Fig. 3C, D). Furthermore, overexpression of *JcGA20ox1* caused a significant decrease in the expression of the downstream floral meristem genes *JcAP1*, *JcFUL* and *JcLFY*, whereas overexpression of *JcGA2ox6* caused a significant increase in the expression of the floral meristem genes *JcAP1*, *JcFUL* and *JcLFY* (Fig. 3E-G). In addition, the expression of the flowering repressor *JcSVP* clearly increased in the *JcGA20ox1*-OE transgenic lines (Fig. 3H). The expression of *JcTFL1s*, which are antagonistic to *JcFT*, was not significantly altered in the *JcGA20ox1*-OE transgenic lines, but the expression of *JcTFL1s* was significantly decreased in the *JcGA2ox6*-OE transgenic lines (Fig. 3I-K). These findings suggest that *JcGA20ox1* may decreased the expression of *JcFT* and its downstream floral meristem identity genes in *J. curcas*, thereby inhibiting floral transition and leading to a late-flowering phenotype in *JcGA20ox1* transgenic *J. curcas*.

To further investigate the effect of GA-regulated floral transition on

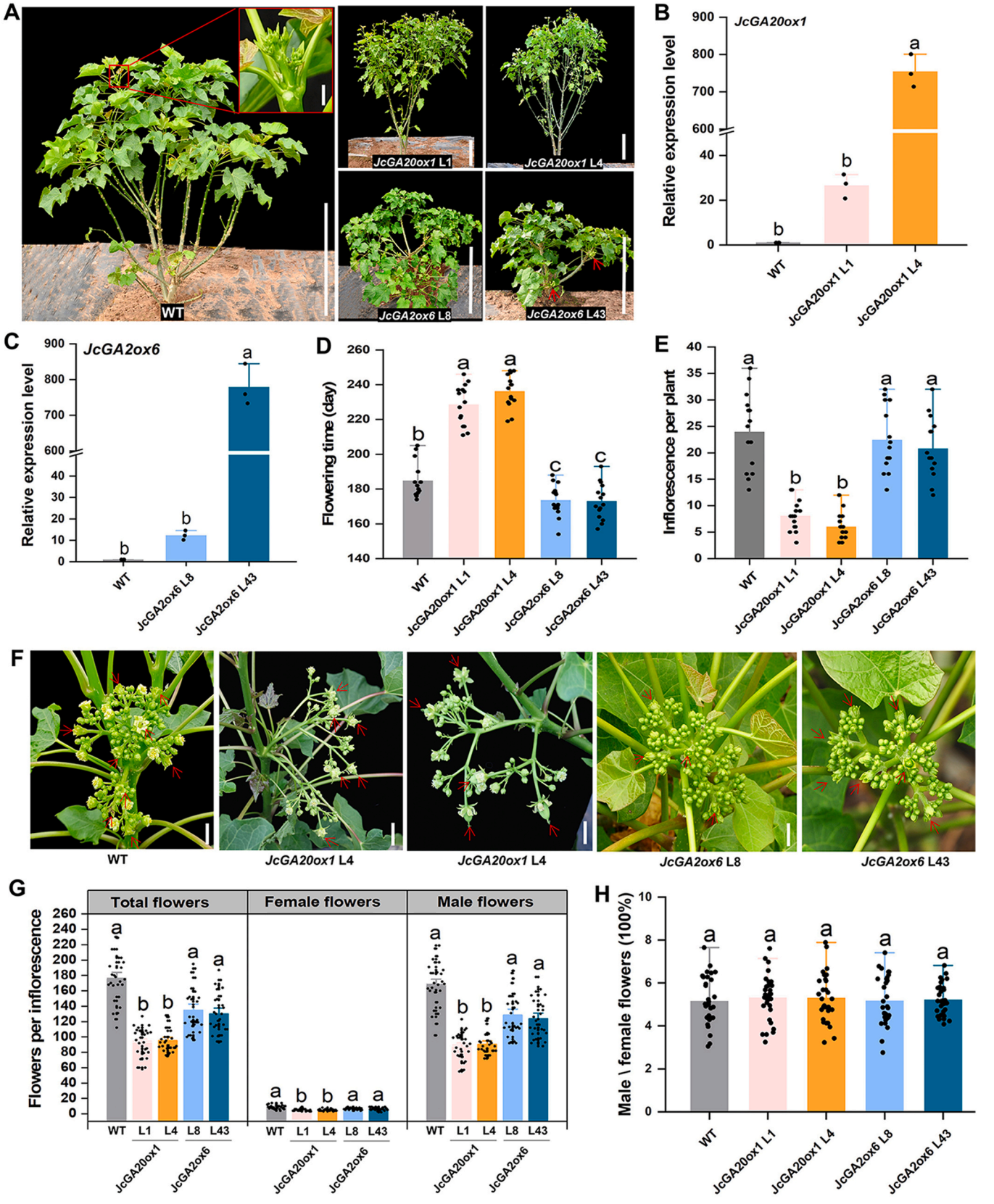

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Fig. 2. Overexpression of *JcGA20ox1* represses flowering, while overexpression of *JcGA2ox6* promotes flowering in *J. curcas*. (A) Phenotypes of the WT, *JcGA20ox1*-OE and *JcGA2ox6*-OE plants. Compared with WT plants, *JcGA20ox1*- and *JcGA2ox6*-overexpressing transgenic lines showed early and late flowering, respectively. Plants were subsequently grown in the field for 7 months. The insets show a close-up of the inflorescences. Arrows indicate inflorescences. Scale bar = 50 cm for plants; scale bar = 1 cm for inflorescences. The plants were grown in the field for 7 months. The insets show a close-up of the inflorescence. Arrows indicate inflorescence. Scale bar = 50 cm for plants; scale bar = 1 cm for inflorescences. (B-C) qRT—PCR was used to measure the expression levels of *JcGA20ox1* (B) and *JcGA2ox6* (C) in the young leaves of the WT, *JcGA20ox1*-OE and *JcGA2ox6*-OE plants. Three biological replicates were subjected to qRT—PCR assays. The results were normalized to those for *J. curcas JcACTIN1*, and the *JcGA20ox1* and *JcGA2ox6* expression levels in the WT were set to 1. mRNA was extracted from seven-month-old plants. (D-E) Comparisons of flowering time (D) and inflorescence number (E) among the WT, *JcGA20ox1*-OE and *JcGA2ox6*-OE plants. Flowering time was measured from germination to bolting. The inflorescence number was measured by recording the total number of inflorescences per plant in one year. (F) Inflorescence phenotype of plants of each genotype; scale bar = 1 cm; the arrows indicate female flowers. (G) Number of flowers per inflorescence in the WT, *JcGA20ox1*-OE and *JcGA2ox6*-OE plants. For B and C, the data are presented as the mean ± SD (n = 3). For D and E, the data are presented as the mean ± SD (n = 15). For G, the data are presented as the mean ± SD (n = 30). Single data points are plotted. Letters indicate significant differences between genotypes according to one-way ANOVA with Tukey's HSD post hoc test ($P<0.05$).

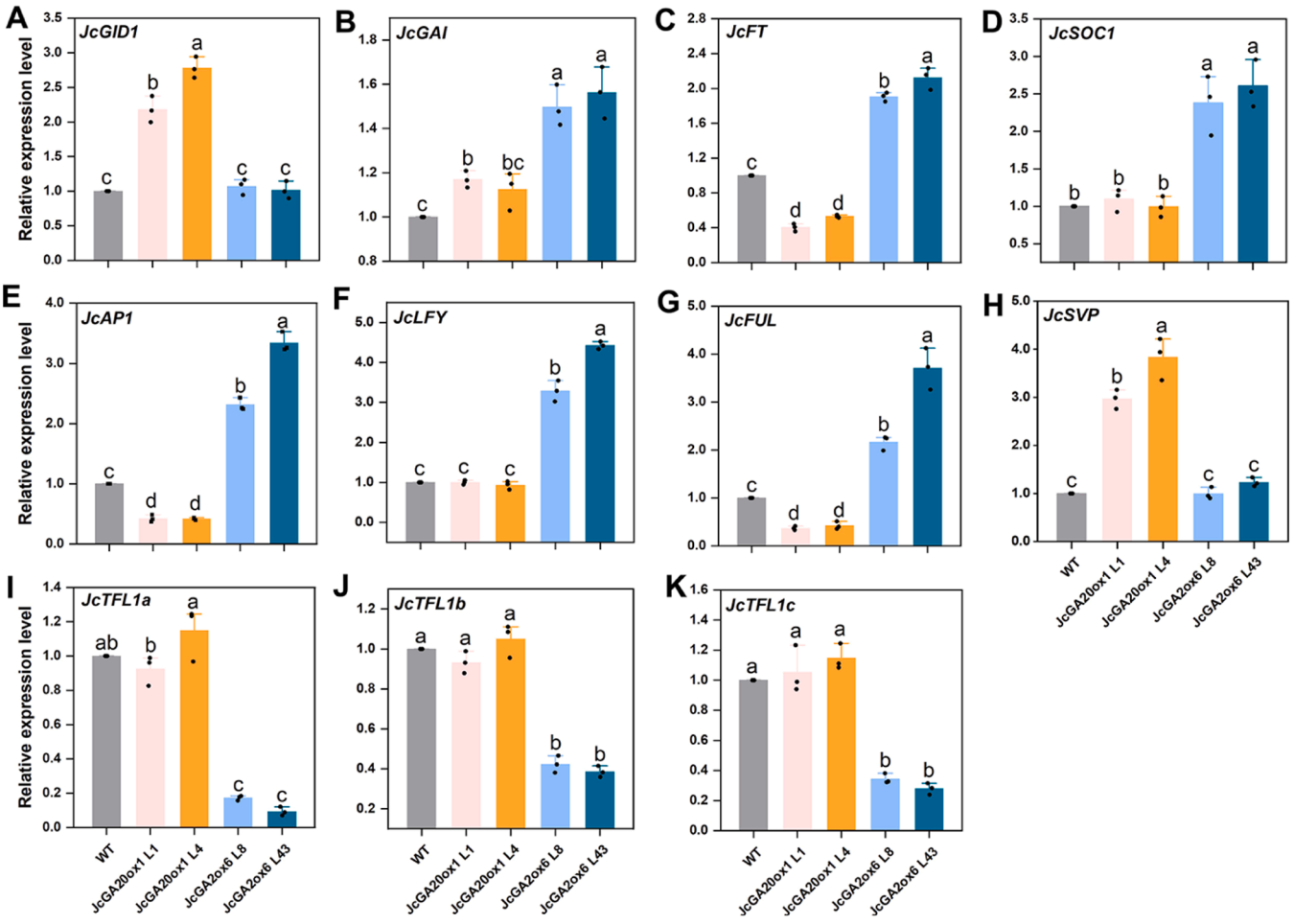

Fig. 3. Changes in the expression levels of flower-related genes in *JcGA20ox1*-OE and *JcGA2ox6*-OE *J. curcas*. The expression levels of flowering-related genes in the *JcGA20ox1*- and *JcGA2ox6*-overexpressing transgenic lines and the WT were determined. Three biological replicates were subjected to qRT—PCR assays. RNA was extracted from inflorescence bud samples. The results were normalized to those of *J. curcas JcACTIN1*. For A—K, the data are presented as the mean ± SD (n = 3 independent samples). The data points are shown individually. Letters indicate significant differences between genotypes according to one-way ANOVA with Tukey's HSD post hoc test ($P<0.05$).

flowering genes, we treated WT inflorescence buds with 1 g/100 mL GA_{4+7}, and then detected the expression of flowering-related genes in the inflorescence buds at 0, 6, 12, and 24 h after treatment by qRT-PCR. The flowering promoting genes, *JcLFY* and *JcAP1*, were up-regulated for the first 6 h and then continuously down-regulated after treatment (Fig. S3C and D). The flowering suppressor gene, *JcSVP*, was up-regulated for the first 12 h and then continuously down-regulated after treatment (Fig. S3F). The floral repressor genes, *JcTFL1a* and *JcTFL1b,* were up-regulated for the first 6 h, and then down-regulated, but up-regulated again at 12 h after treatment (Fig. S3G and H). Surprisingly, the expression of *JcFT*, *JcSOC1*, *JcFUL* and *JcTFL1c* did not change significantly at 24 h after treatment (Fig. S3C and D). In conclusion, we observed that exogenous GA_{4+7} treatment affected the expression of some flowering genes in a short time, but the process of change was more complex than that in the transgenic plants.

3.4. *JcFT rescues the flowering inhibition of JcGA20ox1*

Overexpression of *JcFT* significantly promoted floral transition and increased the number of inflorescences in *J. curcas* (Li et al., 2014; Bai et al., 2022). In this study, we found that the expression of *JcFT* decreased in the *JcGA20ox1*-OE transgenic plants (Fig. 3C).To validate

the role of *JcFT* in the GA flowering pathway, we performed a hybridization complementation experiment in which the *JcFT*-OE plants were used as paternal plants and the *JcGA20ox1*-OE L4 plants as maternal plants. Afterwards, 15 hybrid plants were obtained (Fig. S2A). In addition, to compensate for the negative effect of the reduced number of female flowers on yield due to the overexpression of *JcGA2ox6*-OE, we also overexpressed the *JcGA2ox6* and *JcFT* genes simultaneously in 15 hybrid plants using *JcFT*-OE and *JcGA2ox6*-OE plants as the parental parents (Fig. S2B). The qRT—PCR results showed that the *JcGA20ox1* × *JcFT* hybrid plants presented high levels of *JcGA20ox1* and *JcFT* expression (Fig. 4A, C), and the *JcGA2ox6* × *JcFT* hybrid plants presented high levels of *JcGA2ox6* and *JcFT* expression (Fig. 4B and C). Overexpression of *JcFT* rescued the late-flowering phenotype caused by *JcGA20ox1*, and an early-flowering phenotype was found in hybrid plants. *JcGA20ox1* × *JcFT* hybrid plants produced inflorescence buds approximately 17 days after germination (Fig. 4D, E). Moreover, the number of inflorescences was also increased in the *JcGA20ox1* × *JcFT* hybrid plants. The *JcGA20ox1* × *JcFT* hybrid plants had 30 more inflorescences than did the WT plants, and 45 more inflorescences than did the maternal *JcGA20ox1*-OE L4 transgenic plants (Fig. 4F). Taken together, these results indicate that *JcFT* can restore the flowering inhibition phenotype of *JcGA20ox1* plants and that hybridization with *JcFT* can promote flowering and increase the number of inflorescences of *JcGA20ox1* transgenic plants. On the other hand, the floral transition time of the *JcGA2ox6* × *JcFT* hybrids was dramatically prolonged, and the number of inflorescences of the hybrids was also significantly greater than that of the WT and parental plants (Fig. 4F).

3.5. *Flowering in the JcGA20ox1-OE J. curcas* × *J. integerrima hybrid is inhibited by overexpression of JcGA20ox1*

To further demonstrate the flowering inhibition caused by overexpression of *JcGA20ox1* in *J. curcas* plants, we used *JcGA20ox1*-OE *J. curcas* transgenic plants (designated *Jc* (*JcGA20ox1*-OE)) as the maternal parent for hybridization with wild-type *J. integerrima* plants (designated *Ji*). Five *Jc* (*JcGA20ox1*-OE) × *Ji* hybrid plants were obtained by crossing (Fig. 5A), which showed the expression of the *JcGA20ox1* gene in these hybrids (Fig. 5D). We found that the *Jc* (*JcGA20ox1*-OE) × *Ji* hybrid plants flowered approximately 6 months later than the *Jc* × *Ji* plants; the *Jc* × Ji plants were segregated from the progeny of the *Jc* (*JcGA20ox1*-OE) × *Ji* hybrids (Fig. 5B, C, G). To investigate the mechanism underlying the suppression of flowering in *Jc* (*JcGA20ox1*-OE) × *Ji* plants by *JcGA20ox1*, we subsequently performed semiquantitative PCR and qRT—PCR to assess the expression levels of *JiFT* in *Jc* (*JcGA20ox1*-OE) × *Ji* hybrid plants, using *Jc* × *Ji* plants as the control. The results showed that the expression level of *JiFT* decreased significantly in the *Jc* (*JcGA20ox1*-OE) × *Ji* hybrid plants with ectopic expression of *JcGA20ox1* (Fig. 5E, F), suggesting that ectopic expression of *JcGA20ox1* inhibits *JiFT* expression in the hybrid, resulting in a late-flowering phenotype. In addition, overexpression of *JcGA20ox1*

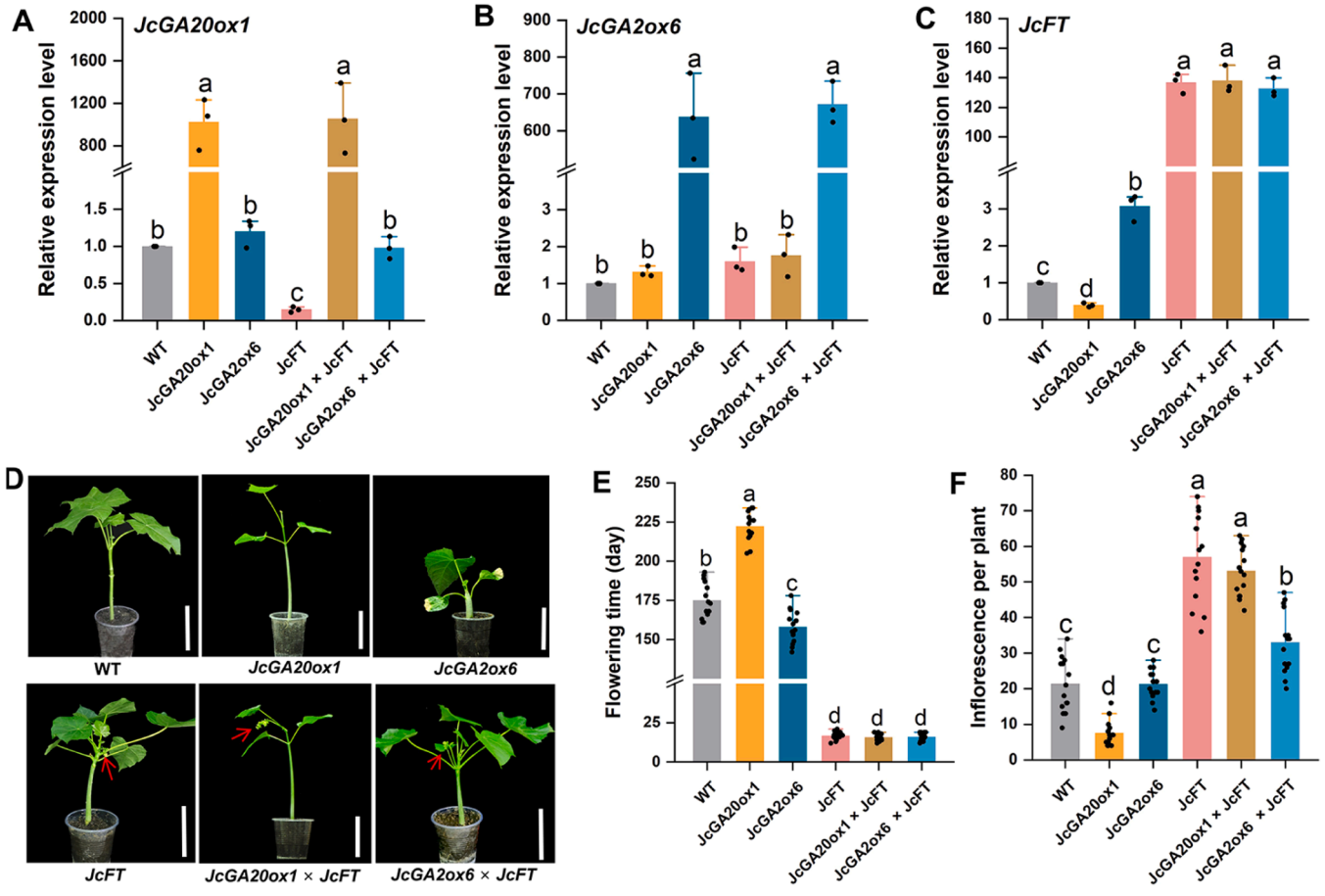

Fig. 4. *JcFT* rescues the flowering inhibition of *JcGA20ox1*. (A-C) The expression levels of *JcGA20ox1* (A), *JcGA2ox6* (B) and *JcFT* (C) in the young leaves of the WT, parental and hybrid plants. Three biological replicates were subjected to qRT—PCR assays. The results were normalized to those of *J. curcas JcACTIN1,* and the expression level in the WT was set to 1. Total RNA was extracted from the leaves of one-month-old plants. (D) One-month-old seedlings of WT, parental and hybrid plants. Arrows indicate inflorescences; scale bar = 8 cm. (E-F) Flowering time (E) and number of inflorescences (F) of the WT, parental and hybrid plants. Flowering time was measured from germination to bolting. The inflorescence number was calculated as the total number of inflorescences per plant in one year. For A, B and C, the data are presented as the mean ± SD (n = 3 independent samples). For E and F, the data are presented as the mean ± SD (n = 15 independent samples). Single data points are plotted. Letters indicate significant differences between genotypes according to one-way ANOVA with Tukey's HSD post hoc test ($P<0.05$).

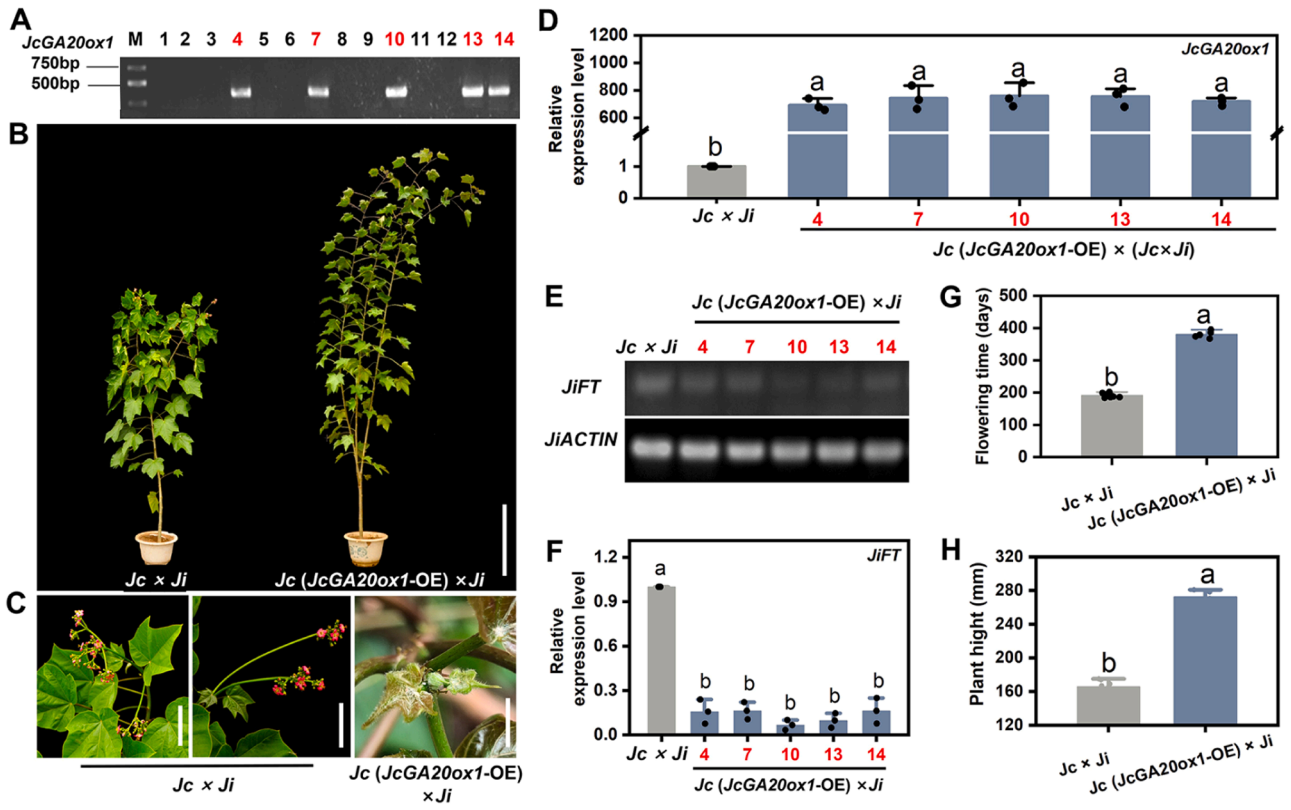

Fig. 5. Flowering in the *JcGA20ox1*-OE *J. curcas* × *J. integerrima* hybrid is inhibited by the overexpression of *JcGA20ox1*. (A) Identification of hybrid lines via PCR analysis of the *JcGA20ox1* gene using real-time PCR primers and promoter-specific primers. The primers XT126 and XB182 were used to amplify a 520 bp fragment (partial sequence of the 35S promoter and *JcGA20ox1* cDNA). (B) Seven-month-old *Jc* × *Ji* plants (left) and *Jc* (*JcGA20ox1*-OE) × *Ji* plants (right) were grown in the greenhouse. Scale bar = 2 cm for plants. (C) Comparison of the growth stages of *Jc* × *Ji* and *Jc* (*JcGA20ox1*-OE) × *Ji* plants. Scale bar=2 cm. (D) qRT—PCR assays of *JcGA20ox1* expression levels in young leaves of *Jc* × *Ji* and *Jc* (*JcGA20ox1*-OE*)* × *Ji* plants. Three biological replicates were subjected to qRT—PCR assays. The results were normalized to those of *J. curcas JcACTIN1*, and the *JcGA20ox1* expression level in the WT was set to 1. (E) Semiquantitative PCR assays of *JiFT* expression levels in young leaves of *Jc* × *Ji* and *Jc* (*JcGA20ox1*-OE) × *Ji* plants. (F) qRT—PCR assays of *JiFT* expression levels in young leaves of *Jc* × *Ji* and *Jc* (*JcGA20ox1*-OE) × *Ji* plants. Three biological replicates were subjected to qRT—PCR assays. The results were normalized to those of *J. integerrima JiACTIN*, and the *JiFT* expression level in the WT was set to 1. (G) Comparison of the flowering times of *Jc* × *Ji* and *Jc* (*JcGA20ox1*-OE) × *Ji* plants. Flowering time was measured from germination to bolting. (H) Comparison of the flowering times of *Jc* × *Ji* and *Jc* (*JcGA20ox1*-OE) × *Ji* plants. The plant height was measured per plant over seven months and recorded. Scale bar=2 cm. For B and E, the data are shown as the mean ± SD (n = 3 independent samples). For C and D, the data are shown as the mean ± SD (n = 9 for *J. integerrima*, n = 5 for the hybrid). Individual data points are plotted. Letters indicate significant differences between genotypes according to one-way ANOVA with Tukey's HSD post hoc test ($P<0.05$).

increased the plant height of the hybrid (Fig. 5B, H).

3.6. *JcFT partially restores the seed yield of JcGA20ox1-OE transgenic plants*

Poor flowering and excessive vegetative buds and leaves contribute to the low yield of *J. curcas*. To obtain a clear picture of the role of the GAs and *JcFT* genes in *J. curcas* breeding, we statistically analyzed the agronomic traits of the WT, transgenic and hybrid plants. We found that overexpression of *JcGA20ox1* in *J. curcas* caused a reduction in the number of fruits per infructescence (Fig. 6D and Fig. S4A), smaller seeds (Fig. 6C, E), a decrease in the ten-seed weight (Fig. 6E), infructescence abortion (Fig. S4B) and the absence of seeds (Fig. S5A), resulting in very low yields of *JcGA20ox1*-OE transgenic plants (Fig. 6G and H). In addition, we found that *JcGA20ox1* negatively regulates infructescence number and seed development. Under normal growth conditions, the inflorescences of the *JcGA20ox1*-OE transgenic plants showed a wilting and withering phenotype approximately 20 days after fertilisation, and the incidence was as high as 43.58% (Supplementary Table 1). Under the influence of the *JcGA20ox1* gene, only 25.63% of the fruits of *JcGA20ox1*-OE plants had three normal seeds, 31.04% and 39.52% of the fruits had two and one seeds, respectively, and 3.81% of the fruits had no seeds (Fig. S5B, C). Taken together, these results indicate that the *JcGA20ox1* gene is not a preferred target for breeding in *J. curcas*. To our surprise, however, crossing with *JcFT* transgenic plants restored the flowering phenotype of 10-seed weights *JcGA20ox1* and caused a significant increase in the number of inflorescences, thus partially rescuing the low-yield phenotype of *JcGA20ox1* via *JcFT* (Fig. 4E, F and Fig. 6G, H). In contrast to those of the *JcGA20ox1*-OE and *JcGA20ox1*× *JcFT* plants, the overexpression of both *JcGA2ox6* and *JcFT* also reduced the number of fruits per infructescence (Fig. S4A); however, fruit development was normal, and the seeds did not exhibit an abortive phenotype (Fig. S5A). In addition, the *JcFT*-OE and *JcGA2ox6* × *JcFT* plants had smaller seed sizes and lower 10-seed weights than did the WT and *JcGA2ox6*-OE plants (Fig. 6D-F). Although overexpression of the *JcFT* gene resulted in an increased number of inflorescences in the *JcGA2ox6* × *JcFT* hybrids compared to those in the WT and *JcGA2ox6*-OE plants, the yield of the *JcGA2ox6* × *JcFT* hybrids was still low due to the small seed size and low 10-grain weight (Fig. 4F). In summary, by observing fruit and seed traits, we concluded that *JcGA20ox1* reduces yield through two pathways, one by suppressing flowering, which is dependent on *JcFT*, and the other by affecting tissue and organ development, which is independent of *JcFT*. We also confirmed that the hybridization of *JcGA20ox1*-OE and *JcFT*-OE plants could partially restore the reduced yield phenotype induced by *JcGA20ox1*-OE.

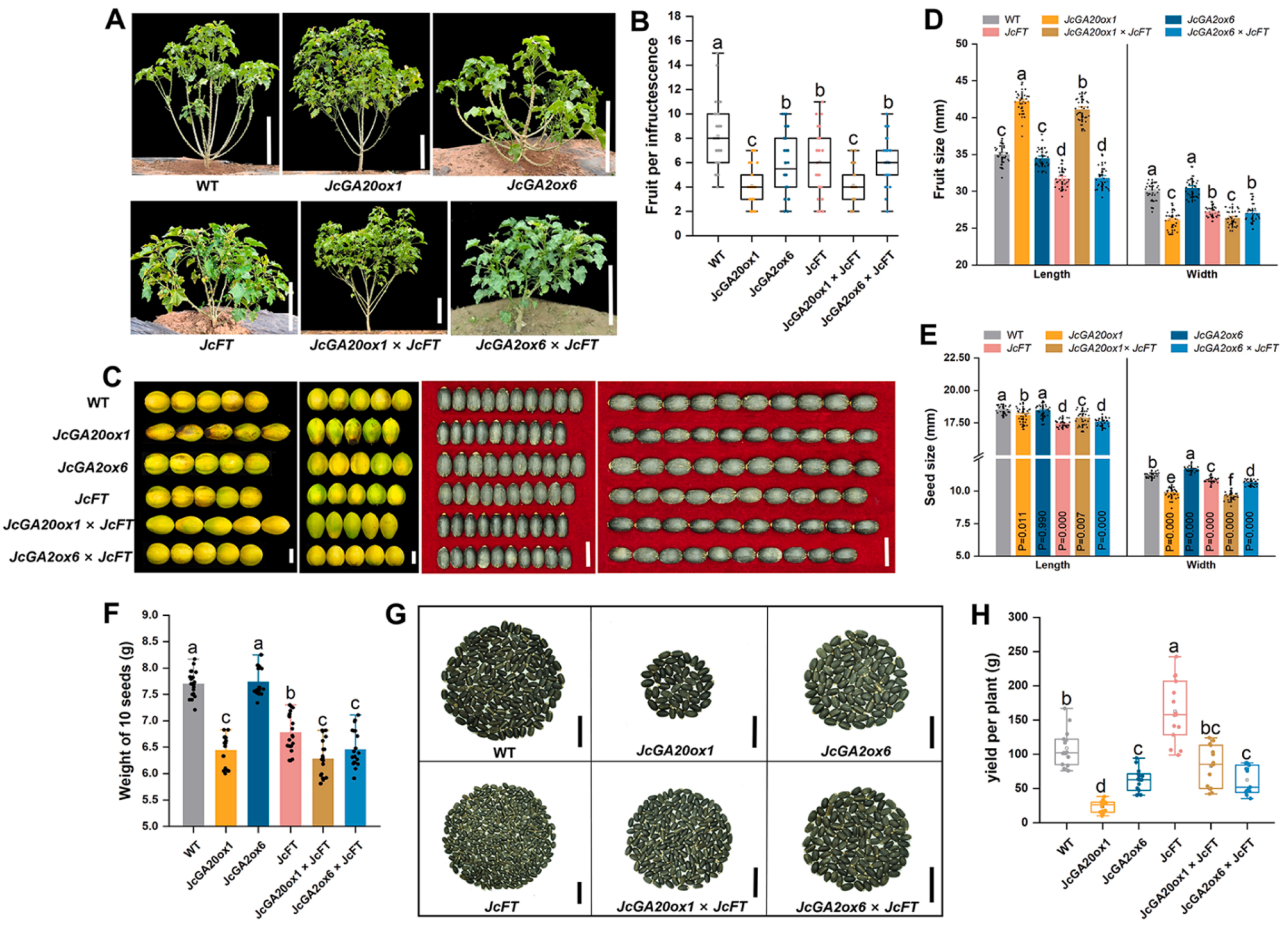

Fig. 6. *JcFT* partially restored the low seed yield of *JcGA20ox1* transgenic plants. (A) Ten-month-old WT, parental and hybrid plants grown in the field; scale bar = 50 cm. (B) Number of fruits per infructescence of the plants of the six genotypets. (C) Fruit and seed morphology of the six plant genotypes. From left to right: fruit length, fruit width, seed length and seed width. Scale bar = 2 cm. (D-E) Fruit size (D) and seed size (E) of the WT plants, parents and hybrids were compared at maturity. (F) The 10-seed weights of the WT, parental and hybrid plants were measured after oven drying. (G) The seeds produced by each of the six genotypes. Scale bar = 5 cm. (H) The seed yield per plant of the six genotypes in the first year was analyzed. For B, F and H, the data are presented as the mean ± SD (n = 15 independent samples). For D and E, the data are presented as the mean ± SD (n = 30 independent samples). Single data points are plotted. Letters indicate significant differences between genotypes according to one-way ANOVA with Tukey's HSD post hoc test ($P<0.05$). Box plots show medians and interquartile ranges of normalized threshold values (), and whiskers and dots show the 1st to 90th percentiles and the remainder of the data points, respectively.

3.7. JcFT cannot restore the seed oil content of JcGA20ox1-OE transgenic plants

J. curcas is considered to be one of the most promising energy crops due to its good oil quality and high oil content; therefore, improving the seed oil content is also one of the goals of *J. curcas* breeding, and we evaluated the effects of GAs and *JcFT* on the oil content. We measured the seed and kernel oil contents of the WT, transgenic and hybrid plants. The results showed that both GAs and *JcFT* negatively regulated the oil content. The average seed oil contents of the *JcGA20ox1*-OE, *JcGA2ox6*-OE and *JcFT*-OE transgenic plants were 29.84%, 31.05% and 28.31%, respectively, which were significantly lower than those of the WT plants. In addition, the oil contents of both the *JcGA20ox1* × *JcFT* and *JcGA2ox6* × *JcFT* hybrids were 27.72% and 27.73%, respectively (Fig. 7A). Furthermore, the seed kernel oil content and seed oil content of the parent and hybrid plants were similar, and all were lower than those of the WT plants (Fig. 7B). These results suggest that *JcGA20ox1*, *JcGA2ox6* and *JcFT* negatively regulate oil synthesis in *J. curcas* such that *JcFT* cannot restore the seed oil content of *JcGA20ox1*-OE transgenic plants.

3.8. JcGA20ox1 reduces yield through seed kernel malformation and reduces oil yield by affecting endosperm development

Our results showed that overexpression of both *JcGA20ox1* and *JcFT* affected endosperm development, whereas overexpression of *JcGA2ox6* did not. Our observation that overexpression of *JcGA20ox1* and *JcFT* resulted in aberrant seed kernel development, as evidenced by overall decreased seed size, and some aberrations in seed development, and that the hybrids obtained were also phenotypically consistent with the parents (Fig. 8A and B), explains another reason for the lower yield of the *JcGA20ox1*-OE transgenic plants. To investigate whether the *JcGA20ox1*, *JcGA2ox6*, and *JcFT* genes affect seed oil content by influencing endosperm development, endosperm cell morphology examined in the WT, parental and hybrid plants. We then found that *JcGA20ox1*, *JcGA2ox6*, and *JcFT* all affected endosperm cell morphology and that *JcGA20ox1* caused the seed endosperm cells to be larger than those of the WT, *JcGA2ox6* and *JcFT* plants, resulting in smaller endosperm cells (Fig. 8C, D). The endosperm cells of the *JcGA20ox1* × *JcFT* hybrids were morphologically similar to those of the *JcGA20ox1*-OE transgenic plants, and those of the *JcGA2ox6* × *JcFT* hybrid plants were similar to those of the *JcFT*-OE transgenic plants (Fig. 8C, D). Based on the observations of endosperm cells, we hypothesized that *JcGA20ox1* reduces the oil content by affecting endosperm development.

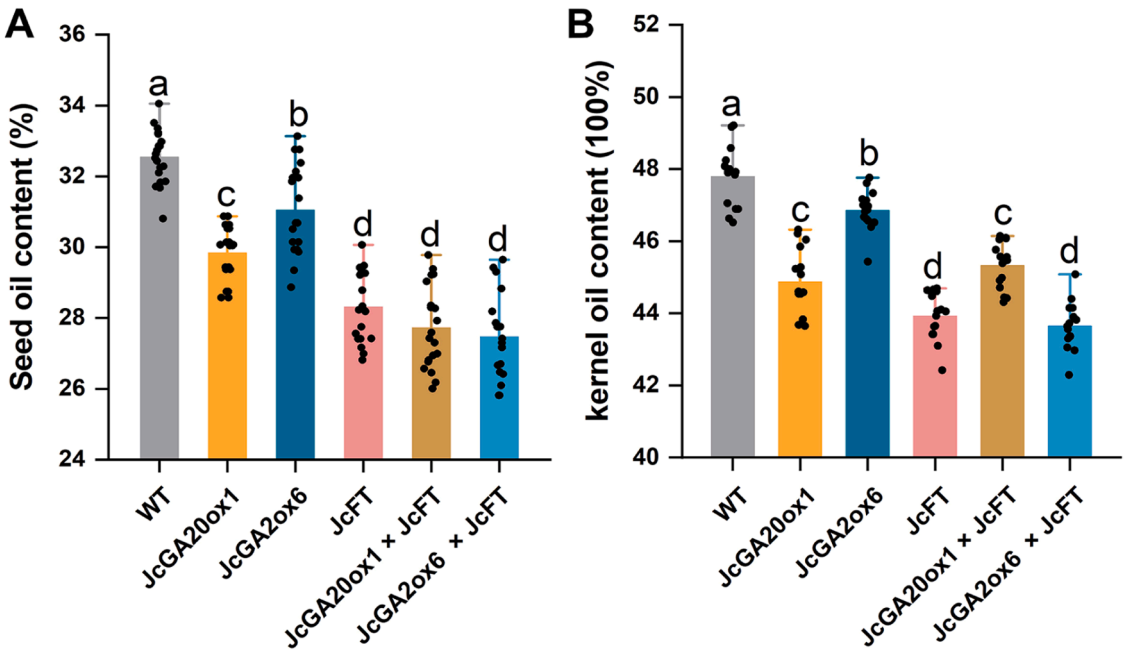

Fig. 7. Seed oil content (A) and seed kernel oil content (B) of the WT, parental and hybrid plants. For A and B, the data are shown as the mean ± SD (n = 20 independent samples). Individual data points are plotted. Individual data points are plotted. Letters indicate significant differences between genotypes according to one-way ANOVA with Tukey's HSD post hoc test (P<0.05).

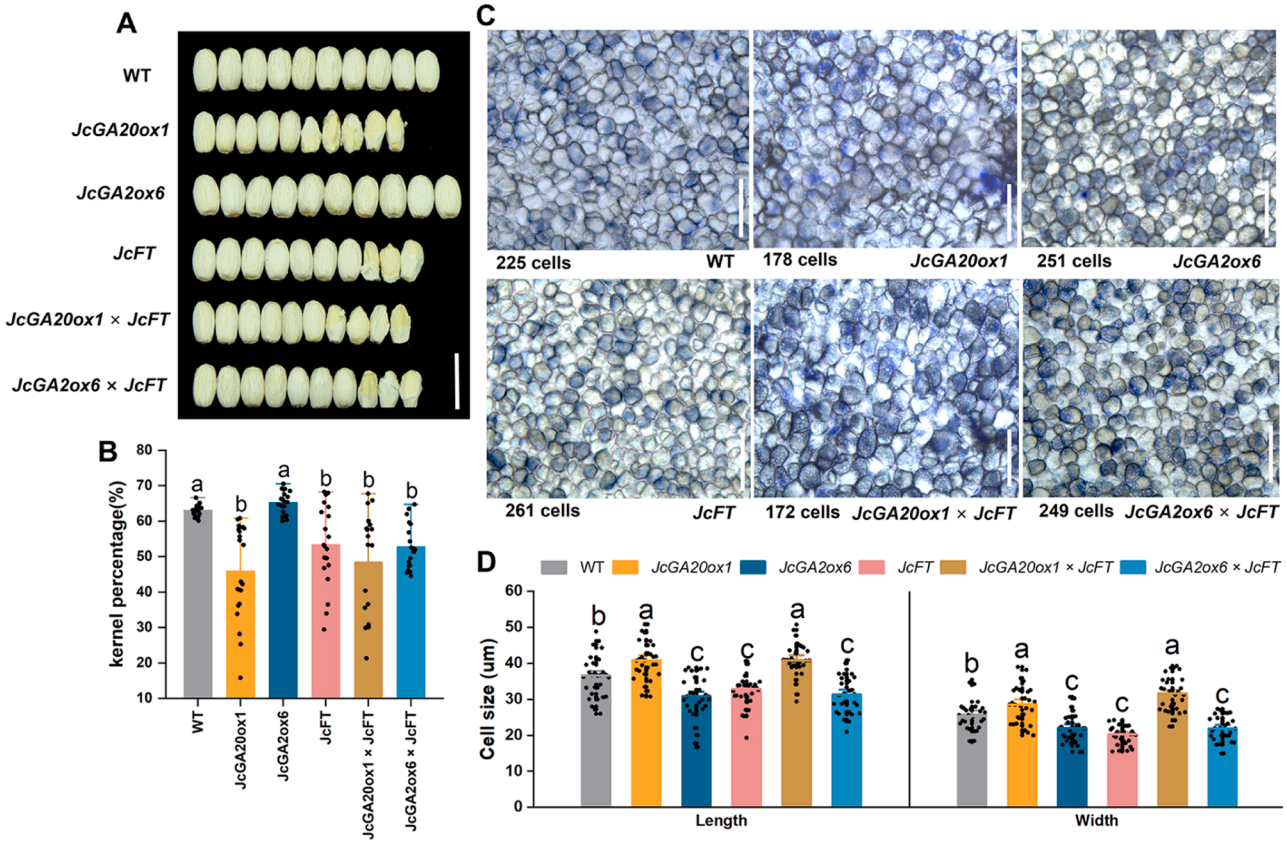

Fig. 8. GA and *JcFT* both negatively regulate endosperm development. (A) Ten seed grains from the six genotype lines were obtained. Scale bar = 2 cm. (B) Hulling percentage (%) of WT plants, parents and hybrids. (C) Kernel cell morphology of the six plant genotypes. Scale bar=100 μm. For A, the data are presented as the mean ± SD (n = 10 independent samples). For A, the data are presented as the mean ± SD (n = 10 independent samples). (D) Endosperm cell size was compared between the WT, parental and hybrid plants. (F) The 10-seed weights of the WT, parental and hybrid plants were measured after oven drying. For B, the data are presented as the mean ± SD (n = 20 independent samples). For D, the data are presented as the mean ± SD (n = 30 independent samples). Single data points are plotted. Letters indicate significant differences between genotypes according to one-way ANOVA with Tukey's HSD post hoc test (P<0.05).

4. Discussion

4.1. Gibberellin affects flowering time and flower development in J. curcas

The involvement of GAs in the regulation of flowering is highly conserved in higher plants, and the divergence is that GAs affect flowering in a species-dependent manner: they promote flowering in annuals and inhibit flowering in perennials. The involvement of GA in flower regulation is highly conserved in higher plants; the difference is that the way in which GA affects flowering depends on the species involved: GA promotes flowering in annuals such as *Arabidopsis* (Eriksson et al., 2006), *rice* (Tamaki et al., 2007) and *Lolium temulentum* (King et al., 2006) but inhibits the transition to flowering in woody perennials such as plums (Bradley and Crane, 1960), apple (Wilkie et al., 2008), avocado (Salazar-García and Lovatt, 1998), *Citrus sinensis* (Muñoz-Fambuena et al., 2012), and sweet cherry (Lenahan et al., 2006). However, one study reported that exogenous treatment with GA_{4+7} promoteds flowering in the *Pinaceae* family (Pharis et al., 1987). Although *Pinaceae*, plums, apple, avocados, *Citrus sinensis* , and sweet cherry are all perennials, *Pinaceae* is an old perennial belonging to gymnosperms phyla that are not flowering plants, while the others are more advanced angiosperms phyla. The two groups of plants may have evolved different reproductive transformation mechanisms in adapting environments, although however, this speculation needs to be further verified in perennial gymnosperms. In addition, PAC enhances reproductive development in woody perennials such as mango (Winston, 1992) and *Litchi chinensis* (Menzel and Simpson, 1990). These studies provide guidance on the validity of exploring the role of GA floral transition in other woody plants. In *J. curcas*, exogenous GA_3 treatment of adult *J. curcas* stem tips invariably inhibited floral transition, conversely, PAC treatment promoted floral transition; furthermore, when GA_3 was treated simultaneously with PAC, GA_3 attenuated the floral-promoting effect of PAC (Li et al., 2018). In our experiments, we investigated the role of GA_{4+7} in the *J. curcas* floral transition by soil application and obtained results consistent with those of exogenous GA_3 treatment (Fig. 1A-C). In summary, we have shown that in addition to GA_3, GA_{4+7} also inhibits floral transition of *J. curcas*.

4.2. Effects of JcGA20ox1 and JcGA2ox6 on flowering time and floral development

GA equilibrium is achieved by the precise regulation of two kinds of pivotal enzymes, *GA20ox* and *GA3ox*, which catalyze the final step of GA biosynthesis, and *GA2ox* which counteracts GA biosynthesis (Olszewski et al., 2002). GA-activated oxidase mutations or DELLA protein mutations promote early flowering in *Arabidopsis* (Yamaguchi, 2008). The *GA20ox* and *GA2ox* gene families are also present in *J. curcas*, and transgenic plants overexpressing *JcGA20ox1* under the control of the 35S promoter and *JcGA2ox6* under the manipulation of the weakly constitutive *JcUEP* promoter were generated in our previous experiments (Ni et al., 2015; Hu et al., 2017); however, the flowering time of the T0 generation of *JcGA2ox6*-OE transgenic plants has not yet been systematically analyzed. After determining the flowering time, it was found that the *JcGA20ox1*-OE plants flowered late and that the *JcGA2ox6*-OE plants flowered early. In other words, increasing the endogenous GAs level inhibited flowering in *J. curcas*. These findings are consistent with those of transgenic phenotypes in other woody plants. In summary, we demonstrated the inhibitory effect of GA on the floral transition in perennial woody plants at both exogenous and endogenous levels (Fig. 2A, D). Our findings are consistent with those of finding in other species, such as avocado (Salazar-García and Lovatt, 1998), *Citrus sinensis* (Muñoz-Fambuena et al., 2012), sweet cherry (Lenahan et al., 2006), and grapevine (Boss and Thomas, 2002).

In addition to floral transition, GA affects flower development in annual herbaceous and perennial woody plants (Khryanin, 2002). GA not only regulates flower initiation and floral organ development but is also essential for sex differentiation (Pimenta Lange et al., 2012). Mutants that are mildly deficient in GA exhibit impaired male fertility due to abnormal stamen development (Khryanin, 2002), while extreme GA deficiency also leads to female sterility (Goto and Pharis, 1999). These phenotypes are currently found in *Arabidopsis* (Khryanin, 2002), walnut (Hassankhah et al., 2018), and *Cucumis sativus* (Choudhury and Phatak, 1959). In *J. curcas*, exogenous GA treatment altered the number of female flowers in inflorescence buds, the number of total flowers and the male/female flower ratio (Li et al., 2018). In the present study, overexpression of *JcGA20ox1* was shown to reduce the number of inflorescences and florets, whereas overexpression of *JcGA20ox6* had no effect on the number of inflorescences but did reduce the number of florets (Fig. 2E-G). Our results have important implications for studying how endogenous GA alterations affect floral development.

4.3. Inhibition of the floral transition in J. curcas and J. integerrima by JcGA20ox1 is associated with decreased the expression of the JcFT and JiFT genes, respectively

In angiosperms, flowering is an important developmental process in the transition from vegetative to reproductive growth that requires precise regulation to maximize reproductive success (Dresselhaus and Sprunck, 2012). The six major flowering pathways, as well as the downstream flowering integration genes and the floral meristems, are genetically co-regulated to control this process (Boss et al., 2004). DELLAs have been reported to act in conjunction with *FT*, *SOC1*, *LFY*, *AP1*, and *FUL* in an indirect manner. *FT* transcription is repressed by a DELLA and CO complex (Bao et al., 2020; Wang et al., 2016). Under SD conditions, the non-flowering phenotype of GA mutants can be rescued by *LFY* and *SOC1* (Moon et al., 2003; Blázquez et al., 1998). Furthermore, under non-inductive SD conditions, DELLAs repress *SPLs* and thus *SOC1* and *FUL* expression. However, during flower development, in the primary flower primordium DELLAs bind *SPLs* transcription factors, which activate *AP1* transcription (Yu et al., 2012).

To investigate the flowering network underlying relationships behind GA regulation, we examined the expression of flowering-related genes in the transgenic plants. *JcGA20ox1* was found to down-regulate the expression of the flowering-promoting genes *JcFT*, *JcAP1*, *JcFUL* and *JcLFY* and up-regulate the expression of the flowering-suppressing gene *JcSVP* (Fig. 3C-H). *JcGA2ox6* was found to up-regulate the expression of the flowering promoting genes *JcFT*, *JcSOC1*, *JcAP1*, *JcFUL* and *JcLFY* and down-regulate the expression of the flowering repressors *JcTFL1s* (Fig. 3C-K). Our previous work has also proven that *JcFT*, *JcLFY*, and *JcTFL1s* play important roles in the flowering process of *J. curcas* (Li et al., 2014; Bai et al., 2022; Tang et al., 2022, 2016a; Li et al., 2017); however, there is no significant effect of the *JcAP1* and *JcSOC1* genes in promoting flowering in *J. curcas* (Tang et al., 2016b), which may be related to the fact that we did not obtain transgenic plants with sufficiently high gene expression. In conclusion, we performed a preliminary investigation of the influence of GA on the flowering network. In addition, a complementary genetic complementation experiment was performed to verify the inhibition of flowering by *JcGA20ox1* through *FT* expression in *J. integerrima*, a member of the Euphorbiaceae family. Overexpression of *JcGA20ox1* inhibited the floral transition in *J. integerrima* and led to a decrease in the expression of *JiFT* (Fig. 5C-E, G, H). This is the first demonstration that overexpression of *JcGA20ox1* decreased the expression of *JcFT* and *JiFT*, and delays floral transition.

We suggest that GA_{4+7} may directly down-regulate the expression of flower-promoting genes and up-regulate the expression of flower-suppressing genes in *J. curcas*. We found no significant effect of GA_{4+7} on the expression of the *J. curcas* homologous genes *FT*, *SOCl,* and *FUL*, which is consistent with findings in apple (Zhang et al., 2019), although these three genes were down-regulated in *JcGA20ox1*-OE plants (Fig. 3C, D and G). In contrast to what has been shown for PAC in

J. curcas (Seesangboon et al., 2018), the expression of the homozygous *J. curcas LFY* and *AP1* genes were down-regulated and then up-regulated by GA_{4+7}, although the expression of these three genes was up-regulated in the *JcGA20ox1*-OE plants (Fig. 3E and H). In addition, the effect of GA_{4+7} on the expression of the homologous *J. curcas TFL1a* and *TFL1b* genes was in agreement with what has been reported in apple (Zhang et al., 2019), although neither gene was significantly altered in the *JcGA20ox1*-OE plants (Fig. 3I and J). In conclusion, we found that exogenous GA_{4+7} treatment affects the expression of flower-promoting and flower-suppressing genes in a short period of time through a complex pathway, which is more complex than that in transgenic plants.

4.4. *JcFT* increased inflorescence number and partially restored the yield-suppresing effect of *JcGA20ox1* and *JcGA2ox6*

The number of inflorescences, the ratio of female to male flowers, the rate of fruit set, fruit and seed size, the number of fruit/seeds and developmental stage all affect crop yield. The regulation of fruit and seed development by GA has been compared in several species. The exogenous application of GA to grapes induced seedless berry development, increased fruit size and improved fruit set by increasing sink strength and sugar signaling activity (Lu et al., 2017). GA positively regulates seed size in *Arabidopsis* and *tomato*, and the *ga3ox1* mutant, which is defective in the GA biosynthesis gene *AtGA3ox1*, produces relatively small seeds (Kanno et al., 2016). *Gibberellic acid-stimulated Arabidopsis* 4 (*GASA4*) positively affects *Arabidopsis* seed size and total yield (Roxrud et al., 2007). The GA-deficient tomato mutant *ga-1* exhibited low fresh fruit weight and a reduced fruit number (Groot et al., 1987).

In this study, we demonstrated that *JcGA20ox1* negatively regulates both fruit and seed development in *J. curcas*. Overexpression of *JcGA20ox1* resulted in fruit abortion and a reduced number of fruits (Fig. S4A, B); smaller seeds, lower seed weights and reduced oil contents (Fig. 6C-F, Fig. 7A, Fig. S5A-C) and malformed seeds with reduced oil contents (Fig. 7A, Fig. 8A). Overexpression of *JcGA2ox6* had no effect on the development of infructescence or on seed development, except for a reduction in infructescence and fruit number (Fig. 6B-F, Fig. S4A, B, and Fig. S5A-C). Taken together, these results indicate that the *JcGA20ox1* gene is not a preferred target for breeding in *J. curcas*. In addition, the phenotypes we found were not similar to those found in annual herbaceous plants where GA increases seed yield, and the underlying mechanisms behind this need to be further investigated.

Previous studies have shown that overexpression of *JcFT* strongly promotes floral transition and significantly increases the number of inflorescences in *J. curcas* (Li et al., 2014; Bai et al., 2022), In this study, we focused on the role of *JcFT* in restoring the negative effects on seed yield caused by GA content in *J. curcas*. We found that overexpression of *JcFT* significantly increased seed yield, although it resulted in smaller seeds and lower oil content (Fig. 6G, H). In addition, hybridization of *JcGA20ox1*-OE plants with *JcFT*-OE plants significantly increased the inflorescence number and partially restored the seed yield, although it did not restore the seed abortion and deformity phenotypes, or lower oil content phenotype (Fig. 6A-H; Fig. 8A-B; Fig. S4A, B; and Fig. S5A-C). In addition, we found that *JcGA20ox1*, *JcGA2ox6*, and *JcFT* negatively regulate oil content (Fig. 7A, B). Based on the observations of endosperm cells (Fig. 8C, D), we hypothesized that *JcGA20ox1* reduces the oil content by affecting endosperm development.

4.5. Overexpression of *JcGA2ox6* represses branching, and *JcGA20ox1* promotes branching

GA is often considered an inhibitor of branching, because *Arabidopsis* mutants for GA biosynthesis and perception, and GA-deficient transgenic plants of various species exhibit an increased branching phenotype (Rameau et al., 2015). In contrast to the above studies, some studies have shown that GA promotes branching. In perennial strawberry and *Rosa* spp, GA biosynthesis is required for bud growth (Tenreira et al., 2017; Choubane et al., 2012). Similarly, in the woody species sweet cherry, *Populus tremula* × *P. tremuloides* and *J. curcas*, the application of GA promoted the outgrowth of lateral buds (Zawaski and Busov, 2014; Pan and Xu, 2011). Interestingly, the inflorescence number in *JcGA20ox1*-OE plants was significantly lower than that of *JcGA2ox6*-OE plants; conversely, the inflorescence number in *JcGA20ox1-OE* × *JcFT* plants was significantly greater than that of *JcGA2ox6*-OE × *JcFT* plants (Fig. 4F). Since inflorescence number is positively correlated with branch number, we suspect that the difference in inflorescence number between the two hybrids might be indirectly induced by the function of GA in regulating branching (Fig. S6A and B). As shown previously, in the T0 generations, *JcGA20ox1* positively regulated the number of branches, whereas *JcGA2ox6* negatively regulated branching (Ni et al., 2015; Hu et al., 2017), these effects were inherited in the T1 generations.

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CRediT authorship contribution statement

Jiapeng Ke: Data curation. **Jie Yang:** Data curation. **Yingxiong Hu:** Methodology. **Li Cai:** Funding acquisition. **Chaoqiong Li:** Resources, Methodology. **Jun Ni:** Methodology. **Ming-Yong Tang:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Zeng-Fu Xu:** Validation, Resources. **Ping Huang:** Writing – original draft, Validation, Data curation.

Declaration of Competing Interest

The authors declare that they have no competing financial interests.

Data availability

Data will be made available on request.

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Author contributions

Ping Huang designed the experiments, created the experimental materials, collected and analyzed the data, and wrote the manuscript. Jie Yang and Jiapeng Ke managed plants in the field and collected the data. Li Cai purchased the experimental agents and collected the data. Jun Ni, Yingxiong Hu and Chaoqiong Li obtained the transgenic plants. Zeng-Fu Xu designed the experiments and revised the manuscript, Mingyong Tang designed the experiments, created the hybrid plants and revised the manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.plantsci.2024.112100.

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