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ORIGINAL ARTICLE

Fackel interacts with gibberellic acid signaling and vernalization to mediate flowering in *Arabidopsis*

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Abstract

Main conclusion **Fackel (FK) is involved in the flowering of *Arabidopsis* mainly via the gibberellin pathway and vernalization pathway. This new function of FK is partially dependent on the *FLOWERING LOCUS C* (*FLC*).**

A common transitional process from vegetative stage to reproductive stage exists in higher plants during their life cycle. The initiation of flower bud differentiation, which plays a key role in the reproductive phase, is affected by both external environmental and internal regulatory factors. In this study, we showed that the *Arabidopsis* weak mutant allele *fk-J3158*, impaired in the *FACKEL (FK)* gene, which encodes a C-14 reductase involved in sterol biosynthesis, had a long life cycle and delayed flowering time in different photoperiods. In addition, *FK* overexpression lines displayed an earlier flowering phenotype than that of the wild type. These processes might be independent of the downstream brassinosteroid (BR) pathway and the autonomous pathway. However, the *fk-J3158* plants were more sensitive than wild type in reducing the bolting days and total leaf number under gibberellic acid (GA) treatment. Further studies suggested that *FK* mutation led to an absence of endogenous GAs in *fk-J3158* and *FK* gene expression was also affected under GA and paclobutrazol (PAC) treatment. Moreover, the delayed flowering time of *fk-J3158* could be rescued by a 3-week vernalization treatment, and the expression of *FLOWERING LOCUS C* (*FLC*) was accordingly down-regulated in *fk-J3158*. We also demonstrated that flowering time of *fk-J3158 flc* double mutant was significantly earlier than that of *fk-J3158* under the long-day (LD) conditions. All these results indicated that *FK* may affect the flowering in *Arabidopsis* mainly via GA pathway and vernalization pathway. And these effects are partially dependent on the *FLOWERING LOCUS C* (*FLC*).

Keywords Brassinosteroid · Fackel · Flowering · *FLOWERING LOCUS C* · Gibberellic acid · Sterol · Vernalization

Abbreviations

BL	24-Epibrassinolide
BR	Brassinosteroid
LD	Long-day
FK	FACKEL

B. Huang, P. Qian and N. Gao contributed equally to this work.

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FLC(D)	FLOWERING LOCUS C(D)
FLK	FLOWERING LOCUS KH DOMAIN
GA	Gibberellic acid
PAC	Paclobutrazol
SD	Short-day
SOC1	SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1

Introduction

Flowering is an important process in the transition from vegetative to reproductive growth in plant, and is regulated by intricate networks of both endogenous and environmental cues (Simpson and Dean 2002; Baurle and Dean 2006). In *Arabidopsis*, the identification and characterization of flowering-defective mutants have led to the identification of at least five signaling pathways that co-regulate flowering, such as photoperiod, autonomous floral initiation, vernalization, brassinosteroid (BR) and gibberellic acid (GA) pathways (Boss et al. 2004; Simpson 2004; Amasino 2005; Clouse 2008; Michaels 2009; Li et al. 2010).

Mutations in *CONSTANS (CO), GIGANTEA (GI) and FLOWERING LOCUS T (FT)* cause late-flowering phenotype which are insensitive to photoperiod (Putterill et al. 1995; Koornneef et al. 1998; Kardailsky et al. 1999). FLOWERING LOCUS KH DOMAIN (FLK), LUMINIDEPENDENS (LD), FVE and FLOWERING LOCUS D (FLD) are involved in the autonomous pathway regulating flowering (Lee et al. 1994; He et al. 2003; Ausin et al. 2004; Lim et al. 2004). FRIGIDA (FRI) and VERNALIZATION INSENSITIVEs (VANs) play major roles in conferring a requirement for vernalization to initiate flowering (Johanson et al. 2000; Gendall et al. 2001; Levy 2002; Sung and Amasino 2004). GA signals play a major role in promoting flowering especially under short day (SD) conditions in *Arabidopsis*. Mutants blocked in GA biosynthesis and defective in GA signaling, such as *gibberellin deficient 1* (*ga1*), *gibberellin insensitive* (*gai*), and *spindly* (*spy*), were found to be delayed in flowering (Wilson et al. 1992; Jacobsen and Olszewski 1993; Wilson and Somerville 1995). Recently, several studies demonstrated that these flowering pathways mainly converge on FLOWERING LOCUS C (FLC) or SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1), which are two MADS box transcriptional factors (Michaels and Amasino 1999; Lee et al. 2000; Li et al. 2016). FLC acts as a strong repressor of initiation of flowering upstream of FLOWERING LOCUS T (FT) and SOC1 (Searle et al. 2006). FRI is a positive regulator that increases the *FLC* expression level (Johanson et al. 2000). In addition, many genes involved in vernalization and autonomous pathways can control the epigenetic status of *FLC* chromatin and reduce *FLC* expression, resulting in early flowering (Amasino 2004; Baurle and Dean 2006). A recent report has shown that DELLA proteins interact with FLC to enhance its inhibition ability. GA can promote the degradation of DELLA to reduce the inhibition ability of FLC, leading to early flowering (Li et al. 2016). GA can also positively regulate *SOC1* expression under SD conditions (Moon et al. 2003).

BRs constitute a class of steroid hormones that regulate various aspects of plant development including photomorphogenesis, xylem formation, cell division and elongation (Li 2010). Several studies revealed that BR-deficient and insensitive mutants *det2*, *dwf4*, and *bri1*, showed a late-flowering phenotype, and the *bas1 sob7* double mutant in metabolizing BRs exhibited an early flowering phenotype (Chory et al. 1991; Azpiroz et al. 1998; Turk et al. 2005; Domagalska et al. 2007). The late-flowering phenotype is accompanied by an increase in *FLC* expression (Domagalska et al. 2007). BR signals result in a chromatin modification which requires ELF6 and REF6 (Yu et al. 2008). BR signaling also interacts with GA and abscisic acid (ABA) pathways signals to control flowering time (Domagalska et al. 2010).

In addition, ABA, ethylene, ambient temperature, and light quality are also critical in regulating flowering (Gray 2004; Achard et al. 2007; Lee et al. 2007; Wollenberg et al. 2008; Davis 2009; Kotchoni et al. 2009). Although the major molecular players and pathways regulating floral transition have been identified, further components remain to be discovered.

Sterols play significant roles as components of the cell membrane and as biosynthetic precursors of steroidal hormones in both animals and plants (Clouse 2000; Gilbert et al. 2002; Attard et al. 2009). In a previous report, rapid changes in sterol and phospholipid/sphingolipid composition led to high activity in the floral apex. These processes might play an important role in regulating the initiation of flowering (Hobbs et al. 1996). In *Arabidopsis*, campesterol, which is one species of the final sterols, is the precursor of BRs (Clouse 2000). A number of proteins and metabolites of post-squalene sterol biosynthesis (SQE1, SMT1, CPI1, CYP51A2, FK, HYD1, and SMT2) play additional roles in plant development independent of that of sterols as the precursors of brassinosteroids (Schrick et al. 2000, 2002; Carland et al. 2002; Souter et al. 2002; Kim et al. 2005; Men et al. 2008; Pose et al. 2009). FACKEL (FK) is a C-14 reductase involved in the early steps of sterol biosynthesis. The previously reported *FK* mutant lines, such as *fk-X224* and *fk-J79*, exhibit sterile and/or lethal phenotypes because of severe defects in embryogenesis and post-embryonic development (Jang et al. 2000; Schrick et al. 2000). More recently, Qian et al. (2013) identified a weak mutant allele of *FK* that is

defective in sterol biosynthesis, *fk-J3158*, which displays uncontrolled cell fate commitment and maintenance of the stomatal lineage in *Arabidopsis*. Another *FK* mutant allele *fk-J79*, which is also known as *extra-long-lifespan1* (*ell1*), was originally characterized for its long lifespan. Likewise, *fk-J3158* also displays a late-flowering phenotype. However, the specific mechanisms are still unclear. Here, we provide physiological and genetic data on the weak allele *fk-J3158* to demonstrate that FK acts as an additional pathway involved in GA signaling and vernalization for flowering in *Arabidopsis*.

Materials and methods

Plant materials and growth conditions

The *Arabidopsis thaliana* Columbia-0 (Col-0; seeds obtained from Arabidopsis Biological Resource Center, ABRC, Columbus, OH, USA) was used as a wild-type control. Mutants and transgenic plants used in this study are as follows: *fk-J3158*, *ProFK::FK/fk-J3158, 35S::FK*, *ga1-3(CS3104)*, *flc(SALK_140021)*, *fk-J3158 flc* and *fk-J3158 ga1-3*. The mutation locus in *fk-J3158, ga1-3* and *flc* background was sequenced (for primer sequences, see Table S1). Seedlings were germinated on 1/2 MS agar plates (Murashige and Skoog 1962) and transferred to soil for growth with 16 h light/8 h dark cycles (LD conditions) or 8 h light/16 h dark cycles (SD conditions) in 22 °C. *ga1-3(CS3104)* and *SALK_140021* was obtained from Arabidopsis Biological Resource Center (ABRC, Columbus, OH, USA).

Imaging and measurement

Images of plants were documented using a Nikon COOLPIX 7000 camera. Flowering time was scored as the number of rosette leaves at flowering when the bolt was approximately 1 cm high. A total of 20–60 plants per genotype were analyzed in each experiment. For statistical analysis, values are reported as the mean ± standard deviation, and unpaired Student's *t* test was performed.

GA_3 and paclobutrazol (PAC) treatment

To the plants in soil, the plants were sprayed by a 100 μM GA_3 (Sigma–Aldrich, St. Louis, MO, USA) or 37 mg/l PAC (Sigma) solution with 0.02% Tween20 under LD conditions until bolting (twice in 1 week). To the plants in MS plate, the media was contained different concentrations of GA_3, 1, 10, 50, and 100 μM. To real-time RT-PCR, the seedlings from MS plate were put into 100 μM GA_3 or 10 μM PAC solutions shaken for 2 h in dark.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from the 3rd or 4th rosette leaves of 15-day seedlings using the Plant RNA Kit (Omega Biotek, Norcross, GA, USA) according to the manufacturer's instruction. To analyze *FLC* and *SOC1* gene expression, total RNA was isolated at the 16th hour of 16 h light in LD conditions and 8th hour of 8 h light in SD conditions. One microgram of total RNA was used for the reverse transcription using a PrimeScript RT reagent Kit (Takara Bio Inc., Kusatsu, Shiga, Japan) according to the manufacturer's protocol. qRT-PCR was performed using SYBR Premix EX Taq II (Takara) on a Stratagene Mx3000P PCR instrument (for primer sequences, see Suppl. Table S1). The glyceraldehyde-3-phosphate dehydrogenase C subunit (*GAPC*) gene was used as a control gene for cDNA amount in the real-time PCR.

GUS reporter gene histochemical analysis

The GUS histochemical analysis of reporter gene was performed on generation seedlings of two independent T1 lines. 24 h staining for GUS activity was performed on samples representing various stages of plant development including 3-day-old whole seedling, 25-day-old whole plant, maturing buds and flowers using a GUS staining solution (Jefferson et al. 1987).

Vernalization treatment

Seeds were planted on MS plates for 3 weeks in 4 °C and in dark. These seedlings were transferred to 16 h light/8 h dark cycles (LD conditions) in 22 °C until the leaves became green. Then they were transferred to soil.

Results

The *fk-J3158* mutant in early sterol biosynthesis delays the flowering time

In a previous study, we reported that the mutations of *FK* affected early sterol biosynthesis and resulted in defects in stomatal development and patterning (Qian et al. 2013). Among all the currently available *FK* mutants, *fk-J3158* is the only weak mutant allele because of its fertility in soil and its weak mutant phenotypes in both stomatal development and other organs as compared to the other *FK* mutants (Qian et al. 2013). Based on the multiple defects of *fk-J3158*, it was suggested that *FK* functions in several processes of plant growth and development. For this study, we explored the dwarfed growth and long lifespan

phenotype of *fk-J3158*. The defective development in *fk-J3158* mutant was complemented by the expression of *FK* cDNA driven by the *FK* own promoter (Fig. 1a–c). To further evaluate the function of FK in the regulation of flowering, a *35S::FK* line was created whereby *FK* was over-expressed in Col (Fig. 1d, e). Under LD conditions, the average bolting days of wild type and *fk-J3158* plants were 33.29 and 48.73, and the average total leaf number were 9.72 and 15.25, respectively (Fig. 2a, c, d). The average bolting days of *ProFK::FK/fk-J3158* were similar to Col and earlier than *fk-J3158* (Fig. 2a, c). The average total leaf number of *ProFK::FK/fk-J3158* was significantly less than *fk-J3158* and slightly more than Col (Fig. 2a, d). The *35S::FK* plants bolting with fewer days and leaves (30.57 and 9.82, respectively) compared to wild type under the LD photoperiod conditions, whereas it displayed normal plant growth and development (Fig. 2a, c, d). In order to understand whether FK functions in the photoperiod pathway, the Col, *fk-J3158*, *ProFK::FK/fk-J3158* and *35S::FK* plants were also grown under SD conditions. We found that *35S::FK*, *ProFK::FK/fk-J3158,* and *fk-J3158* plants all exhibited significantly delayed time to bolting and increased total leaf number similar to Col plants (Fig. 2b, e, f). Thus, these results suggested that FK functions to promote flowering and regulates flowering time independent of the photoperiod pathway.

FK is expressed in tissues with high mitotic activity

The *fk-J3158* plants displayed a late-flowering phenotype. Many flowering genes are expressed in tissues with high mitotic activity, especially floral organs, in order to regulate flowering. Previous studies showed that *FK* is strongly expressed in the shoot apical meristem (Jang et al. 2000; Schrick et al. 2000), but its expression in the floral meristem has not been characterized. In order to further analyze the involvement of FK in flowering time, we characterized the organ- and tissue-specific of *FK* expression patterns using *ProFK::GUS* plants. We observed *FK* expresses in the hypocotyl and cotyledons, immature roots, immature leaves, shoot meristem, immature clip, and in the flowers (Fig. 3a–f). In floral organs, the GUS signal was very strong in sepals, anthers, stigma, carpel, and pollen, and absent in petals (Fig. 3e). These results suggested that *FK* is expressed in both the shoot meristem and floral organs to regulate flowering.

BRs are unable to specifically complement the late-flowering phenotype in *fk-J3158*

Sterols are a class of membrane isoprenoid lipids that are essential to regulate embryogenesis and post-embryonic stages of plant development via a currently unidentified signaling pathway in *Arabidopsis* (He et al. 2003; Lindsey et al. 2003; Men et al. 2008; Babiychuk et al. 2008; Qian et al. 2013). It is known that FK functions upstream of BR biosynthesis. Previous studies reported that BRs are important signals to promote the floral transition by repressing the expression of *FLC* (Chory et al. 1991; Azpiroz et al. 1998; Turk et al. 2005; Domagalska et al. 2007). To determine whether specific BRs could rescue the late-flowering phenotype of *fk* mutant plants, seeds of *fk-J3158* were sown on media containing in 1, 10, and 100 nM of 24-epibrassinolide (BL) (Fig. 4a, b). When *fk-J3158* was treated with 1 and 10 nM of BL, the frequency of bolting plants in 27 day were significantly enhanced, suggesting a partial rescue of the late-flowering phenotype in the mutant. However, this rescued flowering phenotype was repressed under the treatment with 100 nM of BL. Moreover, these results were very similar to those seen in

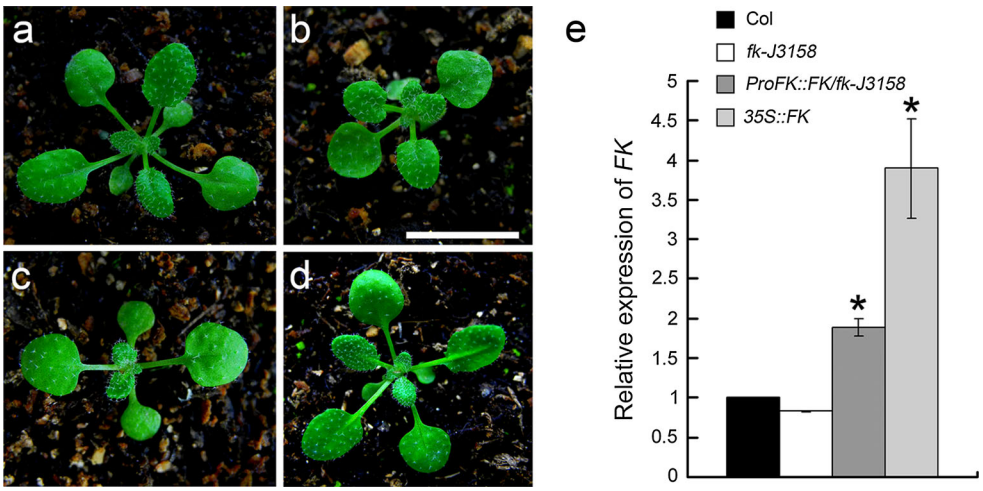

Fig. 1 Seedling phenotypes of Col-0, *fk-J3158*, *ProFK::FK/fk-J3158* and *35S::FK*. Seedling phenotypes in 15-day-old Col-0 (**a**), *fk-J3158* (**b**), *ProFK::FK/fk-J3158* (**c**) and *35S::FK* (**d**). *Bars* 2 cm. **e** *FK* mRNA levels in 15-day-old Col-0, *fk-J3158*, *ProFK::FK/fk-J3158* and *35S::FK*

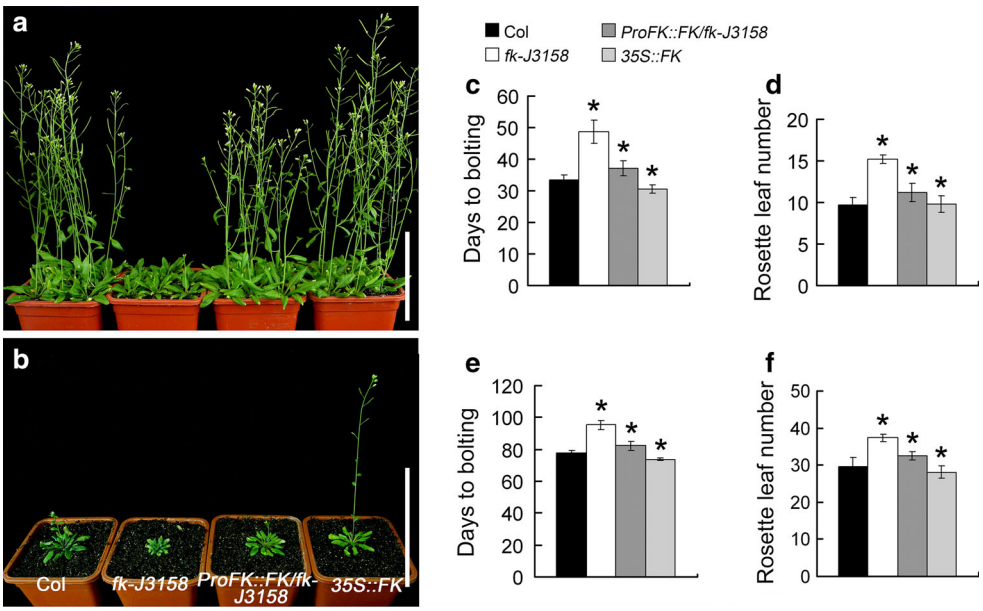

Fig. 2 Phenotypes of flowering in *fk-J3158*, *ProFK::FK/fk-J3158* and *35S::FK* under different photoperiods. 40-day-old plants were grown under LD conditions (16 h light/8 h dark) (**a**), and 55-day-old plants were grown under SD conditions (8 h light/16 h dark) (**b**). From *left* to *right* were Col-0, *fk-J3158*, *ProFK::FK/fk-J3158* and *35S::FK* plants. *Bars* 10 cm. Flowering time was indicated as bolting days (**c**) and rosette leaves (**d**) under LD conditions ($n = 60$). Flowering time was indicated as bolting days (**e**) and rosette leaves (**f**) under SD conditions ($n = 25$). *Asterisk* indicates significant difference from the value of Col-0 (*t* test, $P < 0.05$)

the BL-treated Col (Fig. 4a, b; Suppl. Fig. S1). These data demonstrated that FK, which functions in the early sterol biosynthetic pathway to regulate flowering, may be independent of the BR-signaling pathway, but the final product BR can indirectly affect the flowering of early-step sterol biosynthetic mutants.

Expressions of a number of autonomous pathway regulators are not affected in *fk-J3158*

It is known that FLK, LD, FVE, and FLD are important regulators of *FLC* in the autonomous pathway. Since the expression of *FLC* was up-regulated in *fk-J3158*, the question was posed whether FK regulation of flowering time is dependent on the autonomous pathway? For this, we investigated the expression of *FLK*, *LD*, *FVE*, and *FLD* in the *fk-J3158* mutant by qRT-PCR analysis under the LD conditions. We found that the expression levels of these genes were not significantly changed (Fig. 4c). This result indicated that FK functioning in the early sterol biosynthetic pathway to regulate flowering may be independent of the autonomous pathway.

The late-flowering phenotype of *fk-J3158* is significantly rescued by application of exogenous GA_3

In *Arabidopsis*, genetic and pharmacological experiments implicate GAs as promoters of flowering, particularly under non-inductive SD conditions (Wilson et al. 1992; Jacobsen and Olszewski 1993; Wilson and Somerville 1995). To determine whether the late-flowering phenotype of *FK* mutants could be rescued by exogenous GA treatment, seeds of *fk-J3158* were sown on MS media containing 1, 10, 50, or 100 μM of GA_3. Under both LD and SD conditions, exogenous application of GA accelerated flowering in Col plants (Fig. 5a–c; Suppl. Fig. S2). Interestingly, *fk-J3158* was more sensitive to GA_3 than Col. Under LD conditions, the bolting days of *fk-J3158* had been rescued to the wild-type level under 1 μM GA_3 treatment, and even lower than the Col (Fig. 5a–c). Similar phenotypes were also evident under SD conditions (Suppl. Fig. S2). Concurrently, the expression of *FLC* in *fk-J3158* following GA_3 treatment was rescued to the untreated-GA_3 wild-type level. However, we observed only a little difference in Col following GA_3 treatment (Fig. 5d). To further demonstrate that the delayed flowering phenotype of *fk-J3158* was rescued by GA_3, we sprayed *fk-J3158* plants grown under LD conditions with a 100 μM GA_3 solution. Comparable to the results observed following GA_3 treatment on MS media, the days to bolting and total leaf number were also significantly reduced than those in Col (Fig. 5e–h). In addition, when plants grown under LD conditions were sprayed with 37 mg/l paclobutrazol (PAC; the GA biosynthetic inhibitor) solution, both the Col and *fk-J3158* plants displayed similar degree of increased bolting days and total leaf number (Fig. 5e–h). These

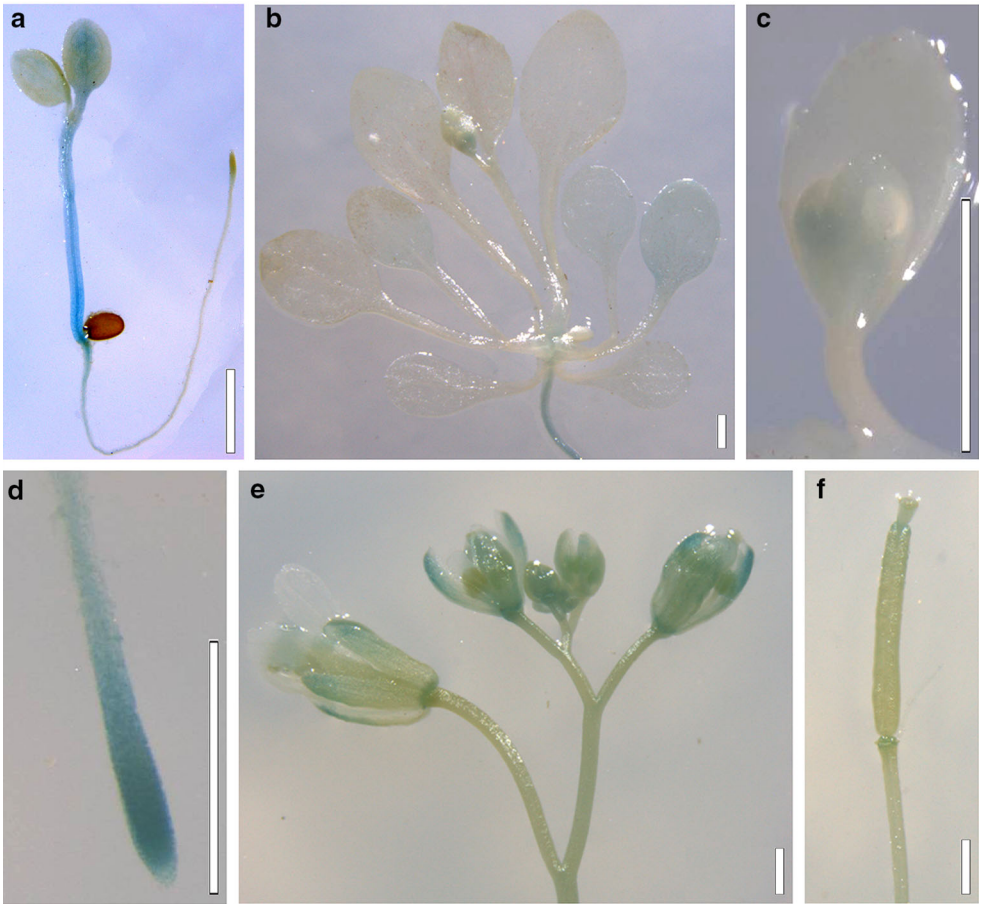

Fig. 3 Expression pattern analysis of *FK*. **a** and **f** Translational fusions of the *FK* regulatory sequences with the GUS reporter gene were examined in transgenic *Arabidopsis* plants. Histochemical staining was performed on 3-day-old seedlings (**a**), 25-day-old plant (**b**), shoot (**c**), root (**d**), floral organ (**e**), and silique (**f**). *Bars* 1 mm

results suggested that the role of FK in the regulation of flowering may be connected to the GA pathway.

FK function and the GA pathway interact with each other in floral transition

Since FK was found to be involved in GA-induced flowering, we wanted to know how FK affected the GA pathway. The expression levels of *GA20ox1*, *GA3ox1* and *GA2ox2* were determined in 15-day-old Col and *fk-J3158* seedling by qRT-PCR. Expression levels of *GA20ox1* and *GA3ox1* were down-regulated in *fk-J3158*, *GA2ox2* expression was up-regulated (Fig. 6a). It is known that GA20ox1 and GA3ox1 promote GA biosynthesis and GA2ox2 assists in GA degradation. Thus, these data suggested that *FK* mutation may lead to an absence of endogenous GAs and an increase in DELLA in *fk-J3158*. A recent report has shown that DELLA proteins interact with FLC to enhance the inhibition ability of FLC (Li et al. 2016). Moreover, *FK* expression was also detected following GA_3 and PAC treatment. We found that *FK* expression was enhanced following GA application but reduced following PAC treatment (Fig. 6b), which is consistent with the results in a previous study (Jang et al. 2000). In the double-mutant analysis, we observed that *ga1-3* could enhance the *fk-J3158* seedling phenotype (Fig. 6c). In addition, no additive effect was observed in the *fk-J3158 ga1-3* double mutant under LD conditions (Fig. 6c–e) and it did not flower during the extended growth period as was observed for *ga1-3*. These results could suggest that GA1 is potentially epistatic to FK in the regulation of flowering. Together, these results suggested that FK function and the GA pathway may interact to regulate floral transition.

The late-flowering phenotype of *fk-J3158* is significantly suppressed by vernalization treatment

Vernalization is an important process that promotes flowering in response to prolonged exposure to low temperature (Gendall et al. 2001; Levy 2002; Sung and Amasino 2004). To determine whether vernalization can prevent the late-

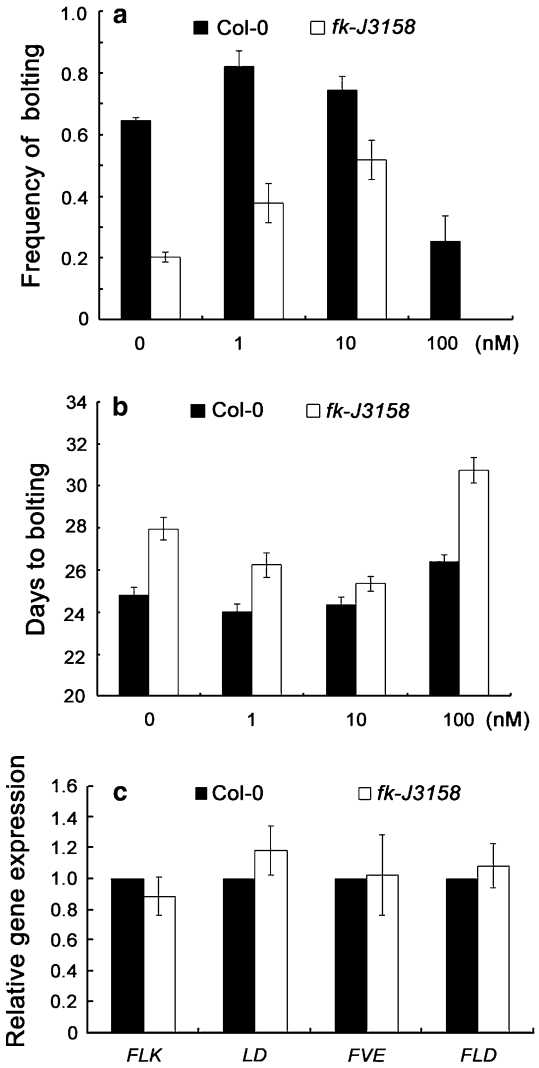

Fig. 4 Dose-response of *fk-J3158* flowering time to different concentrations of BL treatment and expression levels of autonomous pathway regulators. Twenty-five-day-old seedlings of Col-0 and *fk-J3158* were grown on MS plate after BL treatment under LD conditions and the frequency of bolting (**a**) and days to bolting (**b**) were measured ($n = 40$). **c** Transcript levels of *FLK*, *LD*, *FVE*, and *FLD* in 15-day-old seedlings

flowering phenotype of *fk-J3158*, the Col and *fk-J3158* plants were grown at 4 °C for 6 weeks before they were transferred to 23 °C under LD conditions. Under vernalization treatment, the average total leaf number of Col plants was reduced from 12.11 to 10.52. However, the average total leaf number of vernalization-treated *fk-J3158* plants was reduced from 18.34 to 11.44, it was much earlier than the untreated plants. Vernalization-treated *fk-J3158* plants also had a lower average total leaf number than that of untreated Col plants (Fig. 7a). Furthermore, the expression of *FLC* was also analyzed because vernalization is known to repress *FLC* transcription. We found that the expression level of *FLC* was high in the untreated *fk-J3158* plants but was drastically decreased after vernalization (Fig. 7b), which was consistent with the data concerning leaf number. Thus, these results suggested that the role of FK in the regulation of flowering may involve the vernalization pathway.

FK function in regulating flowering is partially dependent on FLC

It is known that several flowering regulation pathways, including the autonomous pathway, vernalization pathway, and BR signaling, converge on the transcriptional regulator FLC that represses the floral transition (Michaels and Amasino 1999; Sheldon et al. 1999; Michaels and Amasino 2001; Domagalska et al. 2007). Many late-flowering mutants have been identified via genetic screens based on high-level *FLC* expression. Thus, we investigated *FLC* expression in the *fk-J3158* mutant using qRT-PCR analysis of plants grown under the LD conditions. As expected, we detected low-level *FLC* expression in Col, but high-level of *FLC* expression in *fk-J3158*. Moreover, *FLC* expression was reduced in *ProFK::FK/fk-J3158* and *35S::FK* plants (Fig. 8a). *FLC* expression levels were consistent with the lifespan of *fk-J3158*, *ProFK::FK/fk-J3158* and *35S::FK* plants (Fig. 8a). FLC can repress *SOC1* expression by directly binding to specific regulatory elements (Searle et al. 2006). Corresponding to *FLC* expression levels, *SOC1* expression was reduced in *fk-J3158* and enhanced in *ProFK::FK/fk-J3158* and *35S::FK* plants (Fig. 8b). The previous experimental, the late-flowering phenotype of *fk-J3158* is significantly rescued by exogenous GA_3 application and vernalization treatment. The expression level of *FLC* was reduced by these same treatments. GA can also promote the degradation of DELLA to reduce the inhibition ability of FLC, which leads to early flowering. To further determine which the late-flowering phenotype in *fk-J3158* was related to FLC, we constructed the double mutant *fk-J3158 flc* to confirm whether *FLC* mutation can rescue the late-flowering phenotype of *fk-J3158* (Fig. 9a). It was found that the flowering time of double mutant *fk-J3158 flc* was significantly earlier than *fk-J3158* but later than Col under the LD conditions (Fig. 9a, b). This result indicates that FK regulation of flowering is partially dependent on FLC.

Discussion

The sterol biosynthetic pathway in *Arabidopsis* has been well described through the identification and characterization of mutants compromised in each biosynthetic step. Previous studies indicate that the sterol biosynthetic pathway plays a role in many different processes including

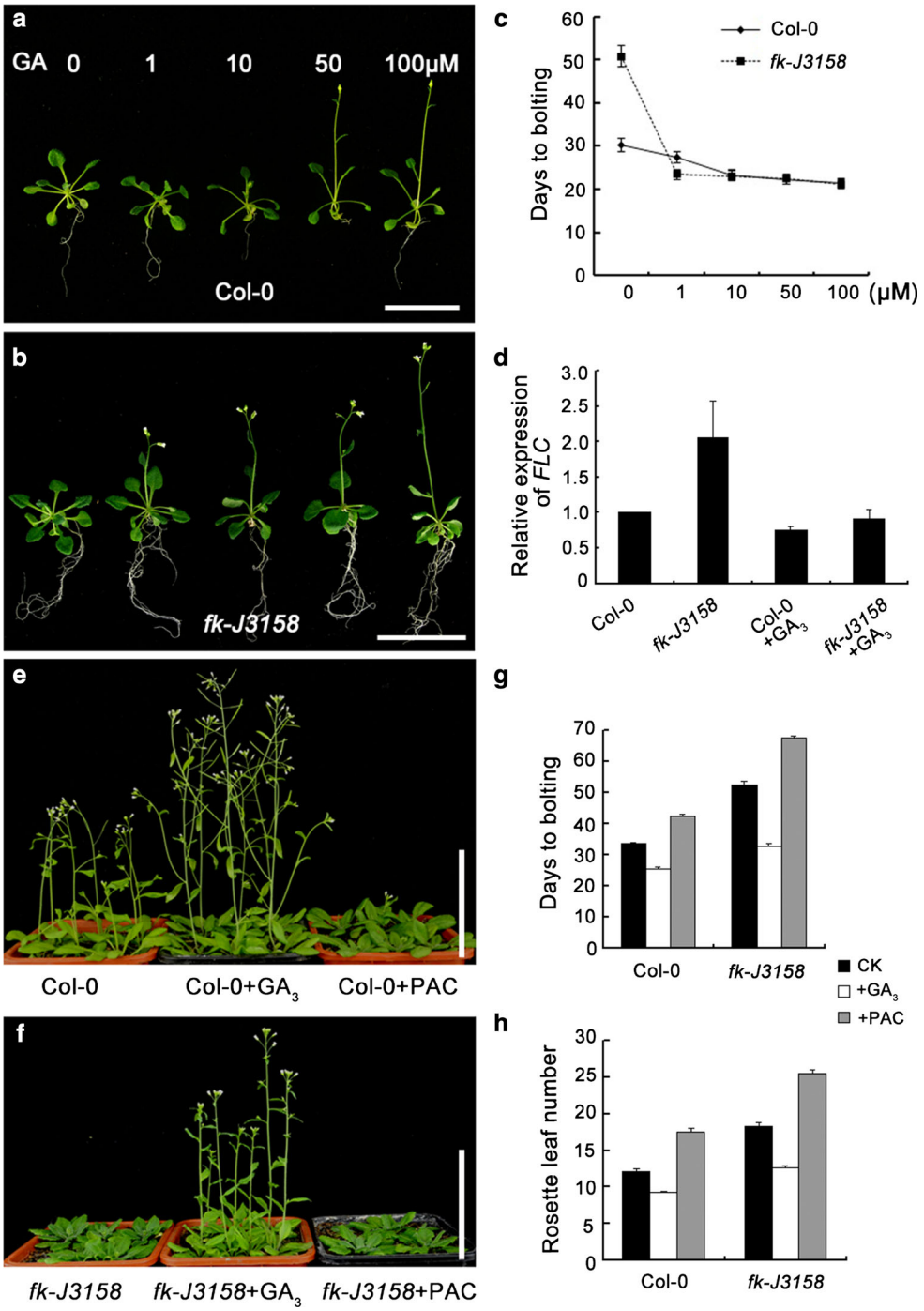

Fig. 5 Response of *fk-J3158* flowering time to GA_3 and PAC treatment under LD conditions. Twenty-five-day-old seedlings of Col-0 (**a**) and *fk-J3158* (**b**) were grown on MS plate after different concentrations of GA_3 treatment under LD conditions. *Bars* 2 cm. **c** Measurement of days after bolting under LD conditions ($n = 40$). **d** Transcript levels of *FLC* in 14-day-old seedlings after GA_3 treatment under LD conditions. Fifty-day-old of Col-0 (**e**) and *fk-J3158* (**f**) plant were grown under LD conditions in soil. Plants were sprayed with 100 μM GA_3 twice a week (+GA_3) or without GA_3 (CK) or with 37 mg/l PAC treatment once a week (+PAC). *Bars* 10 cm. **g**, **h** Measurement of days after bolting and rosette leaf number under GA_3 and PAC treatment. Values represent the mean ± standard deviation ($n = 20$)

morphogenesis, cell differentiation, cell polarity, and cell patterning (Lindsey et al. 2003; Schaller 2003; Boutté and Grebe 2009). BRs are synthesized from the sterols campesterol. It was reported that most BR-biosynthetic mutants and BR-insensitive mutants exhibited a delayed flowering time. The majority of mutants compromised in the sterol biosynthetic pathway show reduced endogenous BR levels and also display late-flowering phenotypes. Because the mutants compromised in early-step sterol biosynthesis display severe multiple defects in plant growth that cannot be rescued by exogenous BR application, it is unknown whether the late-flowering phenotypes in these sterol mutants are dependent on the BR pathway. In this study, the weak *FK* mutant allele *fk-J3158* resulted in plants that displayed late flowering and that were not rescued by exogenous BL treatment, these results suggest that FK or early-step of sterol biosynthesis is involved in an additional pathway that regulates floral transition, which is

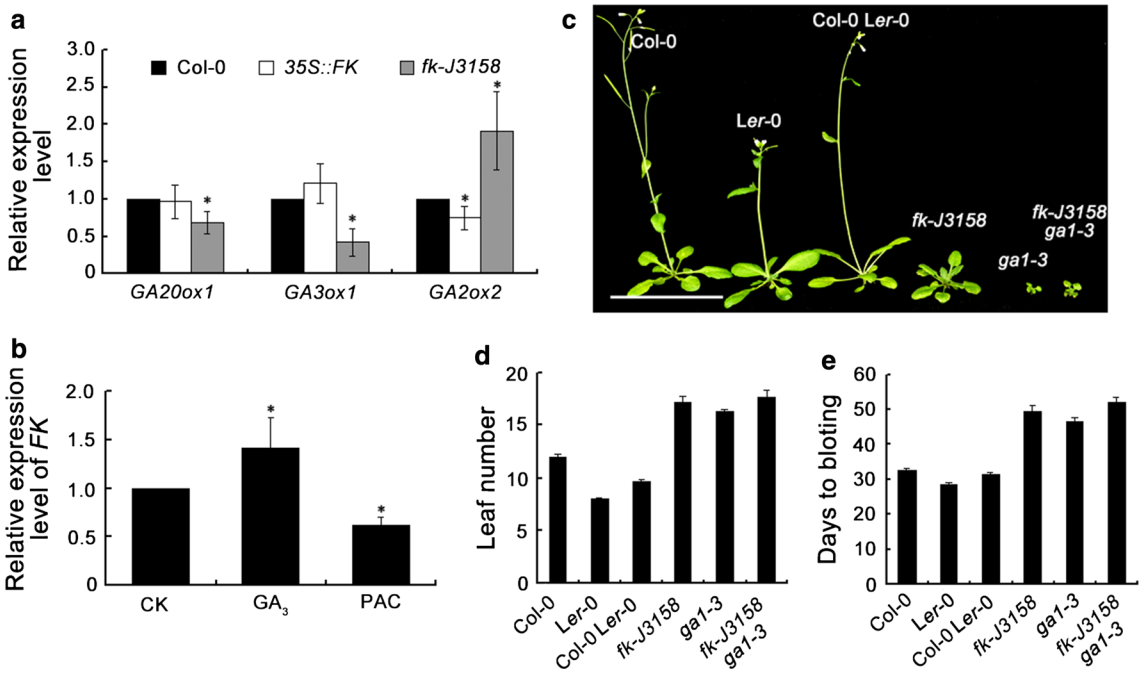

Fig. 6 FK interacts with gibberellin pathway in flowering. **a** Transcript levels of *GA20ox1, GA3ox1, GA2ox2* and *GID1* in 14-day-old *fk-J3158* seedlings under LD conditions. **b** Transcript levels of *FK* in 14-day-old wild-type plant by gibberellins and PAC treatment under LD conditions. *Asterisk* indicates significant difference from the value of Col-0 (*t* test, $P < 0.05$, $n = 5$). **c** Floral-timing phenotypes of 35-day-old Col-0, L*er*-0, *fk-J3158*, *ga1-3*, Col-0 L*er*-0, and *fk-J3158 ga1-3* under LD conditions. *Bars* 4 cm. Total leaf number at bolting (**d**) and days to bolting (**e**) was analyzed in 35-day-old Col-0, L*er*-0, *fk-J3158*, *ga1-3*, Col-0 L*er*-0, and *fk-J3158 ga1-3*

independent of sterols and BRs. Many studies have suggested that there are potentially a number of unidentified intermediates in the sterol biosynthetic pathway that mediate a novel signaling pathway to regulate the development of embryos, vascular tissue, and stomatal development in *Arabidopsis*, in a manner independently of sterols and BRs (Jang et al. 2000; Schrick et al. 2000, 2002; Souter et al. 2002; Kim et al. 2005; Men et al. 2008; Qian et al. 2013). It is possible that the late-flowering phenotypes of early-step sterol biosynthetic mutants are the result of disruptions in the synthesis of these unidentified intermediate molecules.

Several floral-transition signaling pathways have been characterized, including the photoperiod, autonomous, vernalization, BR, and GA pathways. The *fk-J3158* plants were sensitive to variation in photoperiod like the wild type and expression of a number of genes involved in the autonomous pathway were not significantly changed in the mutant plants, suggesting that FK might be not be involved in the photoperiod and autonomous pathway to regulate flowering. Several pathways are activated by epigenetic silencing of *FLC* (Simpson and Dean 2002; Sung and Amasino 2004; Alexndre and Hennig 2008), which can act as a negative regulator of *SOC1*. We found that *FLC* expression was enhanced and *SOC1* expression was reduced in *fk-J3158*. Moreover, following exogenous GAs treatment, the late-flowering phenotype was significantly rescued in *fk-J3158* and the expression level of *FLC* was also reduced to the wild-type level. In addition, the late-flowering phenotype and the *FLC* expression level in *fk-J3158* were also rescued by vernalization treatment. We demonstrated that flowering time in the double mutant *fk-J3158 flc* is significantly earlier than that in *fk-J3158* under the LD conditions. This result indicates that FK regulating flowering is partially dependent on the FLC, and that FK regulates floral transition mainly via FLC that integrates the GA and vernalization pathways.

In a previous study, the GA pathway was found to directly regulate *SOC1* expression, but this is a function in the downstream of *FLC* because GAs did not influence the expression of *FLC*. In a recent study, DELLA was shown to interact with FLC to enhance the inhibition ability of FLC. The inhibition ability of FLC is reduced when exogenous GA promoted the degradation of DELLA (Li et al.2016). This may be one of main reasons that GA promotes flowering. However, in this study, *FLC* expression in *fk-J3158* plants was partially rescued by GA treatment. In other studies, it was reported that overexpression of *GASA5* which is involved in GA signaling, also resulted in high-level *FLC* expression. Moreover, the low expression levels of GA biosynthetic genes suggest that endogenous GAs are absent in *fk-J3158* plants. Based on the fact that the *FK* expression can be regulated by exogenous GAs and PAC, it was suggested that the FK and

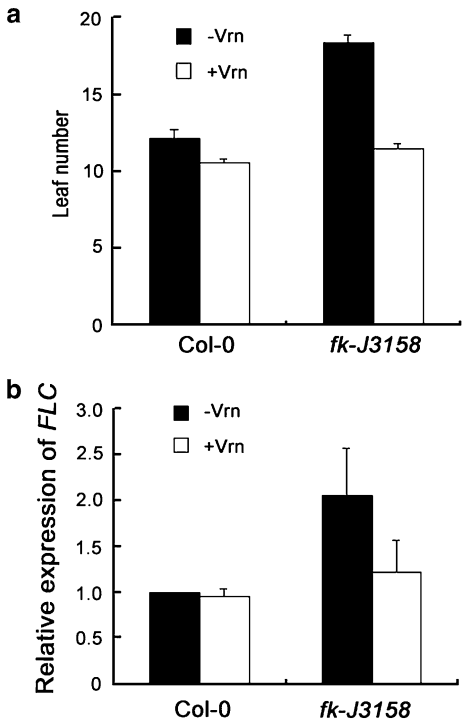

Fig. 7 Leaf number and *FLC* transcript level in *fk-J3158* under vernalization. **a** Measurement of total leaf number for Col-0 and *fk-J3158* mutants under cold-treated (3 weeks) (+Vrn) or untreated (−Vrn). Values represent the mean ± standard deviation ($n = 20$). **b** *FLC* expression in *fk-J3158* mutant after vernalization treatment. Values represent the mean ± standard deviation ($n = 5$)

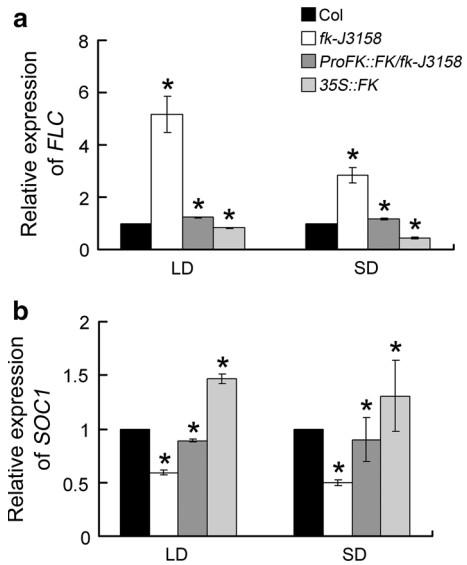

Fig. 8 Analysis of flowering genes *FLC* and *SOC1* in Col, *fk-J3158*, *ProFK::FK/fk-J3158* and *35S::FK* seedlings under LD and SD conditions. **a** Transcript levels of *FLC* in 15-day-old seedlings under LD and SD conditions. **b** Transcript levels of *SOC1* in 15-day-old seedlings under LD and SD conditions. *Asterisk* indicates significant difference from the value of Col-0 (*t* test, $P < 0.05$, $n = 5$)

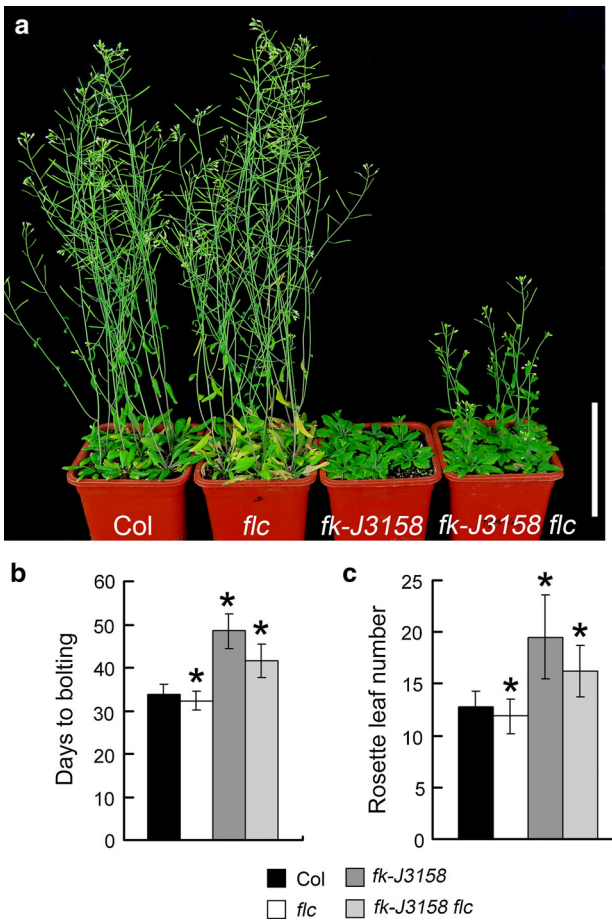

Fig. 9 The phenotype of double mutant *fk-J3158 flc*. **a** The phenotype in 40-day-old Col, *flc*, *fk-J3158*, *fk-J3158 flc* under LD conditions. *Bars* 10 cm. Flowering time was measured as bolting days (**b**) and rosette leaves (**c**) under LD conditions ($n = 30$). *Asterisk* indicates significant difference from the value of Col-0 (*t* test, $P < 0.05$)

GA pathway interact to regulate flowering time and that GA may affect *FLC* expression indirectly. It is known that hormone and environment interaction exists in the regulation of seed germination, in which the levels of GAs can be up-regulated by vernalization to break dormancy and promote germination (Yamauchi et al. 2004). In addition, FLC also play an important role in GA and temperature-dependent germination (Chiang et al. 2009). In the present study, the late-flowering phenotype of *fk-J3158* can be attributed to the high expression level of *FLC* and the low level of GA. Vernalization treatment and GA application can reduce the expression level of *FLC* in *fk-J3158*. This result indicates that FK regulating of *FLC* expression is dependent on both the vernalization and GA pathways. Exogenous GA application also can promote DELLA degradation that reduces the suppression ability of FLC. These factors together rescue the late-flowering phenotype of *fk-J3158* plants. We also demonstrated that the double

mutant *fk-J3158 flc* can rescue the late-flowering phenotype of *fk-J3158*. This result indicates that FK's regulation of flowering is partially dependent on FLC. Therefore, it may be possible that vernalization and GA pathways interact with each other thus affecting floral transition indirectly by regulating FLC. Although it was reported that other mutants in the early steps of the sterol biosynthetic pathway also display a late-flowering phenotype, it would be interesting to test whether these defects could be rescued by GA and/or vernalization treatments.

Author contribution statement BH, PQ, NG and JS conducted experiments and analyzed data. SH, PQ and BH designed the research and wrote the manuscript. All authors read the manuscript and approved the manuscript.

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