Two *FLX* family members are non-redundantly required to establish the vernalization requirement in Arabidopsis

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Abstract

Studies of natural genetic variation for the vernalization requirement in Arabidopsis have revealed two genes, *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*), that are determinants of the vernalization-requiring, winter-annual habit. In this study, we show that *FLC EXPRESSOR LIKE 4* (*FLL4*) is essential for up-regulation of *FLC* in winter-annual Arabidopsis accessions and establishment of a vernalization requirement. *FLL4* is part of the *FLC EXPRESSOR* (*FLX*) gene family and both are non-redundantly involved in flowering-time control. Epistasis analysis among *FRI, FLL4, FLX* and autonomous-pathway genes reveals that *FRI fve* exhibits an extreme delay of flowering compared to *fri fve*, but mutants in other autonomous-pathway genes do not, indicating that *FVE* acts most antagonistically to *FRI*. *FLL4* may represent a new member of a FRI-containing complex that activates *FLC*.

Introduction

Arabidopsis thaliana has been used as a model for studying flowering-time regulation. Major environmental inputs that induce flowering in Arabidopsis and many other plant species are prolonged exposure to cold (vernalization) and photoperiod. There are two types of Arabidopsis accessions, summer-annual and winter-annual. Summer-annual accessions flower rapidly without vernalization, whereas winter-annual accessions require vernalization for rapid flowering[1]. Studies of natural genetic variation have revealed two genes, *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*), that are determinants of the winter-annual habit[2, 3]. *FLC* encodes a MADS-box transcription factor that suppresses flowering[4], and *FRI* encodes a nuclear protein containing coiled-coil domains[5]. *FRI* is essential for *FLC* expression to be sufficiently high to repress flowering in the fall[6]. During vernalization, *FLC* is repressed, resulting in plants which flower in the spring[4, 7]. Summer-annual accessions typically contain naturally occurring mutations in *FRI*, resulting in attenuated *FLC* expression such that flowering occurs without vernalization[6, 8].

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In addition to *FRI*, a range of genes have been identified that are required for the elevated *FLC* expression that confers a winter-annual habit[9]. Some of these genes encode proteins involved generally in chromatin modification and RNA processing. Therefore, loss of these genes results in both a rapid flowering phenotype due to loss of *FLC* activation and pleiotropic phenotypes due to misregulation of other genes. This class includes components of the RNA Polymerase II-associated factor 1 (PAF1) complex (*VERNALIZATION INDEPENDENDE 3* (*VIP3*), *VIP4, EARLY FLOWERING 7*(*ELF7*), *ELF8*)[10, 11, 12], a histone H3K36 methyltransferase (*EARLY FLOWERING IN SHORT DAYS* (*EFS*)[13], histone H3K4 methyltransferases (*ARABIDOPSIS TRITHORAX-LIKE 1* (*ATX1*), *ATX2* and *ATX-RELATED 7* (*ATXR7*))[14, 15], a SWR1-related, nucleosome-remodeling factor (*PHOTOPERIOD INDEPENDENT EARLY 1*(*PIE1*), *ACTIN-RELATED PROTEIN 4* (*ARP4*), *SUPPRESSOR OF FRIGIDA 3* (*SUF3*)/*ARP6*/*EARLY IN SHORT DAYS 1* (*ESD1*)[16, 17, 18, 19], RNA-processing factors (*SERRATE* and *HUA2*)[20, 21] and a mRNA cap-binding protein (*ABA HYPERSENSITIVE 1*(*ABH1*)/*CAP BINDING PROTEIN 80* (*CBP80*))[22].

In contrast to general regulators for which mutations have pleiotropic effects, mutations in other regulators of *FLC* affect only flowering and thus might be specific to the vernalization system. One example is *FRI*, which by genome-wide expression analyses, affects only *FLC* expression[8]. Several mutations in other genes caused rapid flowering by attenuating *FLC* expression without pleiotropic effects[23, 24, 25, 26, 27]. A recent study indicates that the proteins encoded by all of these are members of a multimeric FRI complex[23]; liquid chromatography-tandem mass spectrometry (LC-MS/MS) using 35S-myc:FRI identified FRIGIDA LIKE 1 (FRL1), FRIGIDA ESSENTIAL 1 (FES1), SUPPRESSOR OF FRIGIDA 4 (SUF4) and FLC EXPRESSOR (FLX)[23] as associated with FRI.

One of *FRI* complex components, *FLX*, is a member of a five-gene family containing *FLX, FLX LIKE 1* (*FLL1*), *FLL2, FLL3* and *FLL4*[23]. Although only FLX was identified as a *FRI* complex component using LC-MS/MS, the other family members physically interact with FRI in yeast two-hybrid assay[23]. Furthermore, yeast one-hybrid assays indicated that FLX and FLL1 have transcriptional activity. However, it is not known whether other *FLX* family members play a role in activating *FLC*.

In addition to the *FLC* activators discussed above, genetic screens have identified several vernalization-insensitive mutants which encode *FLC* repressors that participate in the vernalization pathway such as *VERNALIZATION INSENSITIVE 3* (*VIN3*), *VERNALIZATION 1* (*VRN1*), *VRN2* and *VIN3-LIKE 1* (*VIL1*)/*VRN5*[24, 25, 26, 27, 28]. These genes are required to epigenetically silence *FLC* expression via enriching H3K9 and/or H3K27 methylation of *FLC* chromatin. *VIN3* is specifically upregulated during cold exposure, and *VIN3* expression is rapidly extinguished after plants are returned to warm conditions[24].

Here, we report that a *FLX* family member, *FLL4*, is essential for *FLC* up-regulation in winter-annual accessions. Furthermore, we show that only two *FLX* family members, *FLX* and *FLL4* are non-redundantly involved in flowering-time control. Finally, we show that

some putative FRI complex components play a role in flowering-time control in the absence of a FRI. Our findings reveal further molecular insight into the control of vernalization.

Results

Two suppressors alter *VIN3* and *FLC* expression without cold

Previous studies have reported the identification of mutants that suppress the delayed flowering that occurs in the absence of vernalization in the line Col *FRI*, which was generated by introgression of functional Sf-2 *FRIGIDA* allele[4, 29]. We screened additional ethyl methane sulfonate (EMS)-generated mutants in Col *FRI* for rapid-flowering lines that exhibited two properties of vernalized Arabidopsis plants in the absence of cold exposure: reduced *FLC* expression and increased *VIN3* expression. In wild-type, *VIN3* is expressed only after a relatively long period of cold exposure[24]. From 98 lines initially identified as rapid-flowering, we found two independent mutants that exhibited both reduced *FLC* expression and increased *VIN3* expression. These mutants, which we now refer to as *flx-3* and *flx like 4-1* (*fll4-1*) (Fig. 1a, b) were chosen for further study because they appeared identical to Col and to vernalized Col *FRI* (Fig. 1c)—i.e., there were no apparent pleiotropic phenotypes and they exhibited features of vernalized plants in the absence of cold exposure.

Mutations in FLL4 and FLX give rise to suppressor phenotypes

To map the location of the genes responsible for the rapid-flowering phenotype of the two mutants that exhibited vernalization-independent increased *VIN3* and decreased *FLC* expression, the mutants were crossed with a line in which *FRI FLC* had been introgressed into the Landsberg *erecta* (L*er*) genetic background[4]. The F1 generations exhibited delayed flowering, and in the F2 generations, delayed to rapid flowering clearly segregated in a 3 to 1 manner indicating that in each line rapid flowering was a single-gene, recessive trait.

The locations of the mutations were mapped in the F2 generations using a combination of SSLP and dCAPs markers which are polymorphic between Col-0 and L*er*[30]. One mutation was narrowed down to an interval between 24.78 MB and 24.91 MB on chromosome 5. Sequence analysis of this interval revealed a nonsense mutation in the first exon of *AT5g61920, FLC EXPRESSOR LIKE 4* (*FLL4*) (Supplementary Fig. S1a). *FLL4* had not previously been identified as being involved in flowering-time control.

The other mutation mapped on the lower arm of chromosome 2 around 12.09 MB. This region contained a gene known as *FLC EXPRESSOR (FLX)* which had previously been identified as a suppressor of *FRI/FLC*-mediated delayed flowering[23, 31]. Further mapping and sequencing analysis identified that this suppressor line contained a single-base change in the first exon of *AT2G30120/FLX* that resulted in the conversion of an arginine to a lysine residue at amino acid position 176 (Supplementary Fig. S1b). This arginine is conserved among all *FLX* family members (Supplementary Fig. S2).

To simultaneously evaluate whether the rapid-flowering phenotype of the mutants is indeed caused by mutations in *FLL4* and *FLX* and whether there are any effects of overexpression, 35S-driven *FLL4* and *FLX* were transformed into the respective mutant lines and the phenotype assessed. Introduction of 35S-driven *FLL4* in *fll4-1* and 35S-driven *FLX* in *flx-3*

rescued the mutant phenotype in all 12 lines examined for 35S::FLL4 and all 9 lines examined for 35S::FLX (Fig. 2). Also, the *flx* suppressor was crossed to another *flx* allele (*flx-2)* that has been previously described[23]. The F1 plants retained the mutant phenotype indicating that we had identified another allele of *FLX* which we designate *flx-3* (Fig. 2).

Suppression of delayed flowering does not require VIN3

As discussed above, the *fll4* and *flx* suppressors were chosen for further study because they exhibited both increased *VIN3* expression, reduced *FLC* expression, and rapid flowering in the absence of cold exposure. To determine whether the *FLC* suppression and rapid flowering of *fll4* and *flx* is dependent on cold-independent *VIN3* expression, we generated *FRI fll4 vin3, FRI flx vin3,* and *FRI fll4 flx vin3* and measured flowering time in these lines (Fig. 3). There is no difference in the level of suppression of *FRI FLC*–mediated delayed flowering when *VIN3* is present or absent in long days (Fig. 3a, b) or short days (Fig. 3c); thus, *fll4* and *flx* suppression of *FRI FLC*-delayed flowering does not require *VIN3*.

Lesions in other *FLX* family members do not affect flowering

The Arabidopsis genome contains 5 *FLX* family members. The FLX family is plant specific and does not appear to share any motifs with other proteins. Among the 5 members, FLL4 and FLX proteins share 30% identity, and 50% similarity. FLX LIKE 1 (FLL1), FLX LIKE 2 (FLL2), FLX LIKE 3 (FLL3) each have 38%, 26% and 29% identities, and 63%, 41% and 50% similarities with FLX, respectively (Supplementary Fig. S2).

To determine whether lesions in other *FLX* family members can suppress *FRI*-mediated delayed flowering similar to the suppression caused by *fll4* and *flx* mutants, *fll1, fll2* and *fll3* mutants were crossed with Col *FRI* and, from the resulting F2 generation, the flowering behavior of homozygous mutants in the presence of *FRI* was determined. Mutations in these other *FLX* family members have no effect on flowering (Supplementary Fig. S3) as also noted by Choi et al.[23]. Thus, based on single mutant analysis, only 2 members, *FLL4* and *FLX,* appear to be necessary for the winter-annual flowering habit. It is, however, possible that other family members have redundant function and that double mutants could have a flowering phenotype.

FLL4 and FLX have non-redundant roles in flowering

Because only *FLX* has transcriptional activity in a yeast one-hybrid assay, Choi et al.[23] speculated that FLX is the only protein among the 5 FLX family members involved in *FRI/FLC*-mediated flowering-time control. However, Choi et al.[23] also showed that all FLX family members interact with FRI in a yeast two-hybrid assay. Our results clearly show that both FLL4 and FLX have a role in flowering-time control. We took two approaches to determine whether the roles of FLL4 and FLX are redundant or non-redundant. First, we generated *fll4* and *flx* double mutants in the *FRI* background. If FLL4 and FLX both contributed an identical dosage-dependent protein function (redundant roles), then a double mutant might be expected to flower earlier than the single mutants. However, *flx-2/fll4-1* and *flx-3/fll4-1* double mutants were identical with respect to flowering to the single mutants *flx-2* or *flx-3* and *fll4-1* in the *FRI* background under both long days (Fig. 4a) and short days (Fig. 4b). Additionally, the double mutants did not exhibit a further decrease of *FLC*

expression (Fig. 4c). These data are consistent with non-redundant roles in for FLX and FLL4 in flowering time control.

The double mutant analyses, however, cannot rule out a model in which FLL4 and FLX do have redundant roles, but that single mutants drop below the threshold of activity required for the *FRI/FLC*-mediated delay of flowering. In this case, a double heterozygote would be expected to behave like a homozygous single *fll4* or *flx* mutant. However, the double heterozygote *flx-3 FLX; fll4-1 FLL4* in the *FRI* background flowers identical to wild type under both long days (Fig. 4a) and short days (Fig. 4b) arguing against redundancy and a threshold model.

A second approach to address the question of possible redundancy is to overexpress *FLL4* in the *flx* background and to overexpress *FLX* in the *fll4* background; overexpression of one gene rescuing the mutant phenotype of the other would be consistent with redundancy. Overexpression of *FLL4* in *flx-3* and overexpression of *FLX* in *fll4-1* did not rescue the mutant phenotype in long days (Fig. 4a) and short days (Fig. 4b). The overexpression constructs are functional because the *FLL4* overexpression construct rescues the *fll4-1* mutant and the *FLX* overexpression construct rescues the *flx-3* mutant (Fig. 2).

No additive phenotype of *fll4* and *flx* with *fri* and *frl1*

We previously reported that a *FRI* family member, *FRIGIDA LIKE 1* (*FRL1*), has a non-redundant role with *FRI* in the establishment of the winter-annual habit[32]. When either gene is mutated, the winter-annual habit is lost because *FLC* expression is attenuated.

To evaluate whether there is an additive flowering phenotype in the *fri* and *frl1*, and *fll4* and *flx* mutants, the phenotype of *fri FRL1 fll4, fri FRL1 flx, FRI frl1 fll4, FRI frl flx* and *fri FRL1 fll4 flx* was assessed in long days and short days (Fig. 5a, b). The flowering time of the mutants tested did not show additive flowering suppression relative to the single *FRI* complex component mutants. These data are consistent with FLL4, FLX, FRI and FRL1 all playing critical non-redundant roles in the *FRI* complex.

fll4 and *flx* suppress the delayed flowering of *fve* and *ld*

Andersson *et al*.[31] reported *FLX* as a flowering regulator based on the mutant phenotype in the C24 accession. A T-DNA knock out mutant, *flx-1*, repressed *FLC* expression, resulting in plants that flower earlier than C24 wild type. In this study, it was reported that *FLX* was not necessary for *FLC* activation when certain autonomous-pathway genes were down regulated. Furthermore, because FLX is part of the *FRI* complex (Choi et al., 2011)[23], and *FRI* is not required for the delayed-flowering phenotype of autonomous-pathway mutants, it might be expected that loss of *FLX* or *FLL4* would not affect the phenotype of autonomous-pathway mutants.

We determined whether *fll4* or *flx* mutations could suppress the delayed flowering that results from loss of function of autonomous-pathway genes, *FPA*[33], *FCA*[34, 35], *FLOWERING LOCUS D* (*FLD*)[36, 37], *LUMINIDEPENDENS* (*LD*)[3] and *FVE*[38]. Accordingly double mutants were made between *fpa-3, fca-9, fld-3, ld-1* and *fve-4* and *flx-3* and *fll4-1*. The delayed flowering of autonomous-pathway mutants *fpa, fca* and *fld* was not suppressed

by mutation of either *fll4* or *flx* (Fig. 6a). However, the delayed flowering of *ld* and *fve* was suppressed by *fll4* and *flx* mutations (Fig. 6a). The *ld* suppression was partial (compare *fri, ld, flx* or *fri, ld, fll4* to *fri* alone), but the *fve* suppression was almost complete. *FLC* expression of the mutant lines was also monitored to determine if the flowering phenotype is a result of reduced *FLC* expression. As shown in Fig. 6b, loss of *FLL4* and *FLX* suppresses delayed flowering of *ld* and *fve* via attenuating *FLC* expression (Fig. 6b). A suppression of the delayed flowering of and *FLC* mRNA levels in *ld* and *fve* also results from lesions in *SUF4*[39].

We further tested whether loss of other *FRI* complex components can suppress the delayed flowering of *fve*. Interestingly, loss of FRL1, FES1 and SUF4 also caused a strong suppression of the delayed flowering of *fve* (Supplementary Fig. S4).

Antagonistic roles of FRI and FVE

To explore the relationship between *FVE* and the *FRI* complex, we compared the flowering time of *fve* and certain other autonomous-pathway mutants with or without *FRI*. Interestingly, *FRI fve* exhibits a more extreme delay of flowering compared to *fri fve*, whereas other autonomous-pathway mutants *fld, ld, fca* and *fpa* did not exhibit such a large difference with or without *FRI* (Fig. 6c). The flowering time of autonomous mutants with or without *FRI* was also correlated with their *FLC* expression level (Fig. 6d). These data are consistent with a model in which *FRI* and *FVE* are acting oppositely on a similar molecular aspect of *FLC* regulation. *FVE* is thought to act by reducing the levels of histone acetylation[38, 40], which would attenuate *FLC* expression. *FRI* increases the levels of *FLC* histone modifications that promote active chromatin[13, 23]. Thus the opposing activities of FRI and FVE may be at the level of chromatin modification at the *FLC* locus. In the absence of FVE function, FRI activity might result in a "hyper activated" chromatin state at *FLC* which is reflected in strongly delayed flowering and increased *FLC* mRNA levels.

In a *FRI* background, loss of one of many putative *FRI* components is sufficient to reduce *FLC* expression to levels as low as those in rapid-cycling wild types that lack *FRI*[32, 39, 41]. As discussed above, however, loss of *FVE* provides a "sensitized" background in which *FRI* causes a stronger delay in flowering. To further explore the roles of *FLL4* and *FLX* in this sensitized background, we made double, triple and quadruple mutants among *fri, fve, fll4* and *flx*. First, we compared the flowering behavior of *fve fll4*, *fve flx* and *fve fll4 flx* with or without *FRI*. The flowering of *FRI fve fll4* and *FRI fve flx* was delayed compared to *fri fve fll4* and *fri fve flx* (Fig. 7a). Thus, in the sensitized *fve* background, *FRI* causes a modest delay of flowering, in the absence of *FLL4* or *FLX*. We also investigated the effect of these genetic combinations on *FLC* expression. The flowering behaviors were clearly correlated with *FLC* expression (Fig. 7b). These data indicate that the ability of *FRI* to delay flowering in the absence of *FLL4* or *FLX* is mediated via *FLC* expression.

We also investigated weather or not *FRI* has activity when other putative complex components are mutated in the *fve* background (Fig. 7c). The flowering time of *FRI fve frl1*, *FRI fve fes1* and *FRI fve suf4* was further delayed compared to *fri fve frl1, fri fve fes1* and *fri*

fve suf4, respectively. Thus, as was the case with *fll4* and *flx, FRI* can cause a modest delay of flowering in the absence of *FES1, FRL1* and *SUF4* when *FVE* is lacking.

Discussion

Our results demonstrate that two *FLX* family members, *FLL4* and *FLX*, have non-redundant roles in *FLC* activation and the establishment of a vernalization requirement. *FLL4* and *FLX* appear to be in the same flowering "pathway" because *fll4* and *flx* single mutants fully suppress the delayed flowering of a *FRI*-containing winter-annual line and the double mutant did not show any additional flowering-time change compared to the single mutants (Fig 4a, b). That FLL4 and FLX have non-redundant roles in flowering is supported by the following evidence. First, as noted above, the *fll4 flx* double mutant has a phenotype identical to the single mutants. If there were redundancy, then the double mutant would be expected to have a more severe phenotype than the single mutants unless both single and double mutants fell below a threshold level of activity required to delay flowering. However, there is no flowering phenotype in the double heterozygote, *fll4-1 FLL4*; *flx-3 FLX*, arguing against a threshold model. Further evidence that FLL4 and FLX have non-redundant roles is that overexpression of *FLX* does not rescue a *fll4* mutant and overexpression of *FLL4* does not rescue a *flx* mutant.

A model that accounts for non-redundancy, but similar function, is that both FLL4 and FLX are non-redundant members of a protein complex. This model is consistent with the results of Choi et. al.[23] who showed that FRI and FLX are part of a multisubunit complex (the *FRI* complex) that is involved in *FLC* up-regulation. In a yeast two-hybrid assay, Choi et. al.[23] showed that all *FLX* family members can interact with FRI. Our results indicate that a fully functional *FRI* complex requires both *FLX* and *FLL4*.

It is intriguing that studies of the molecular basis of the vernalization requirement in Arabidopsis reveal at least two examples of the involvement of non-redundant members of a gene family. Specifically, both *FRI* and the *FRI* family members *FRL1* or *FRL2*[32, 42] and, as shown in this study, both *FLX* and the *FLX* family member *FLL4* are necessary to create a vernalization requirement. The *FRI* and *FLX* gene families are present in all sequenced plant genomes including tropical species that do not have a vernalization requirement, and vernalization systems appear to have evolved independently and relatively recently in different plant lineages as an adaptation to temperate climates[9]. Thus, vernalization was not likely to be the ancestral role of the founding members of these gene families. If the ancestral role of such genes involved multimerization, then gene duplication could permit an additional copy to encode a protein that continues to function in the multimer complex along with the original member, and this would permit a version of the complex to diverge such that the diverging complex could assume a new function such as elevating *FLC* expression to create a vernalization requirement. Consistent with this evolutionary path to a vernalization requirement, FLX, FRI and FRL1 reside in the same FRI complex[23] and it is likely that FLL4 will be found in this complex as well.

There are some intriguing differences in phenotype when different autonomous-pathway mutants are combined with *FRI*. *FRI fve* exhibits an extreme delay of flowering compared to

fri fve, but *FRI fld, FRI ld, FRI fca* and *FRI fpa* do not, and the flowering time of these genotypes correlates with their *FLC* expression (Fig. 6a,b). *FVE* is an Arabidopsis homolog of the human histone-binding proteins *Retinoblastoma-Associated protein 46* and *48*. As a component of a histone deacetlyase (HDAC) complex, *FVE* has been shown to be involved in a vernalization-independent system of *FLC* repression[38, 40, 43, 44, 45]. Thus, perhaps the *FRI* complex and its components such as FLL4 and FLX act antagonistically to FVE by increasing histone acetylation at *FLC*. Antagonistic functions would account for the strong delay of flowering caused by *FRI* in the *fve* mutant. Interestingly, it has been reported that the product of the autonomous-pathway gene *FLD* is also part of an FVE-containing HDAC complex[43, 44, 45]. However, our result that *FRI fld* does not exhibit the enhanced delayed flowered exhibited in *FRI fve* indicates that FVE and FLD act in separate pathways. This is consistent with previous reports that *FVE* functions independently of *FCA, FPA* and *FLD* to repress *FLC* expression via various loss/gain of function mutant series[37, 46].

It is also intriguing that, in the absence of *FRI, fll4* and *flx* mutants strongly suppress the delayed flowering of *fve* and, to a lesser extent, that of *ld*, but loss of *FLL4* and *FLX* does not have a strong suppressive effect on other autonomous-pathway mutants. This could indicate that FLL4 and FLX have a function that is independent of the FRI complex if the complex was completely non-function in the absence of any components. Alternatively, in a "sensitized" *fve* mutant background, the FRI complex might have residual *FLC*-promoting activity in the absence of FRI, and loss of *FLL4* or *FLX* would attenuate this residual activity. This residual activity of the FRI complex without *FRI* would be consistent with the evolutionary model, noted above, of FRI evolving a role of "hyperactivating" *FLC* to create a vernalization requirement; for example, there may have been a precursor complex to the current FRI complex that had moderate *FLC*-promoting activity. An *in vitro* system to study the interaction of FRI complex components and FVE with *FLC* would be useful to investigate this model.

Methods

Plant Materials and Growth Conditions

The wild-type Arabidopsis plants used in this study were either wild type Columbia (Col/fri) or a *FRI* introgression line (Col FRI)[4, 29]. Seeds were sown on Perti-plates containing 2 mM MES at pH 5.7, 0.8% (w/v) Phytoblend agar (Caisson Lab, North Logan, UT) and 0.65 gL^{-1} Peter's Excel 15-5-15 CalMag fertilizer. Plated seeds were not stratified and plants were transferred to soil. Plants were grown in Metro-Mix 360 (SUN GRO Horticulture) and fertilized with Peters Excel CalMag (15-5-15) at regular intervals in short days (8 hr lights/16 hr dark), long days (16 hr lights/8 hr dark), or continuous light from cool-white fluorescent tubes (PPFD approximately 60–70 μmol m^{-2} s^{-1}). For vernalization experiments, seedlings on agar were incubated at 4 °C in short days and transferred to soil after cold exposure.

Gene expression analysis

Total RNA was isolated from seedlings using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions, and treated with RQ1 RNase-Free DNase (Promega). DNA-free

RNA was used to generate cDNA using qScript cDNA SuperMix (Quanta Biosciences). After preparation of cDNA, quantitative real time PCR was perfomed using Applied Biosystems 7500 Fast Real-Time PCR System. Ubiquitin 10 was used as an internal control. All primers used are listed in Supplementary Table S1.

Gene sequences and mutants

All sequence data in this article can be found in GenBank Nucleotide Core under the following accession numbers: AT4G00650 (*FRI*), AT5G10140 (*FLC*), AT5G57380 (*VIN3*), At2g30120 (*FLX*), At3g14750 (*FLL1*), At1g67170 (*FLL2*), At1g55170 (*FLL3*) and At5g61920 (*FLL4*). The mutant lines described in this manuscript are: *elf7-2*[15], *elf8-1*[12], *efs-3*[13], *flx-2*[23], *fll1-1* (SALK_139524), *fll2-1* (CS822746), *fll3-1* (SALK_002678), *vin3*[24], *frl1-1*[32], *fpa-7*[47], *fca-9*[22], *fld-3*[48], *ld-1*[49], *fve-4*[47], *fes1-1*[41], *suf4*[39].

Constructs

For *35S::FLL4* and *35S::FLX*, the coding regions of *FLL4* and *FLX* were amplified by PCR, and cloned into D-TOPO entry vectors (Invitrogen) and then moved into pGWB502 vectors (kindly provided from Dr. Tsuyoshi Nakagawa) using the Gateway cloning system (Invitrogen)[50]. All primers used are listed in Supplementary Table S1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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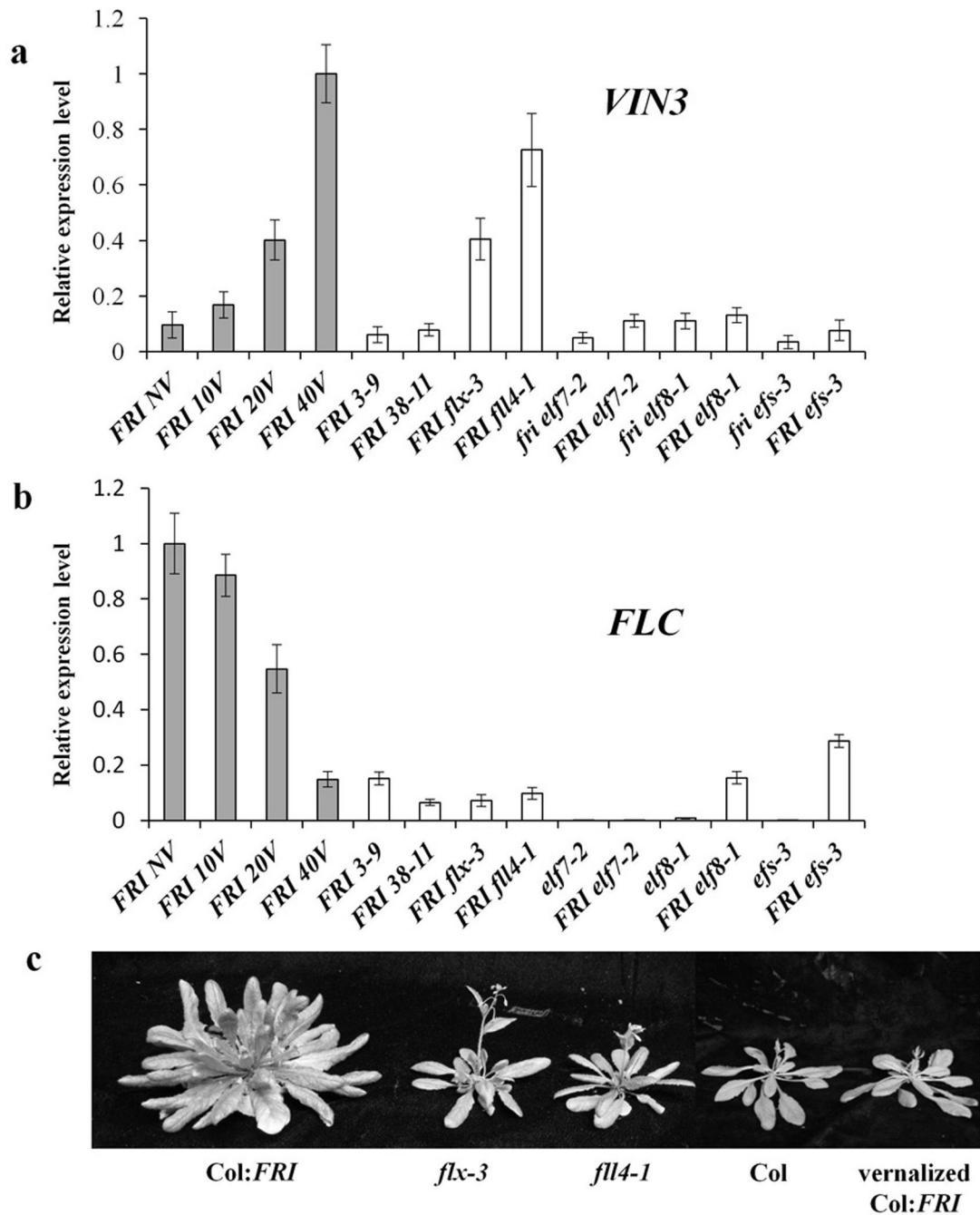
Figure 1. *flx-3* and *fll4-1* alter *VIN3* and *FLC* expression without cold

flx-3 and *fll4-1* exhibit *VIN3* up-regulation (a) and *FLC* down-regulation (b) without vernalization. *FRI 3–9* and *FRI 38-11* were selected as negative controls for *VIN3* expression from among the *FRI*-suppressor mutants we identified. *FLC* and *VIN3* expression were also monitored in *fri elf7-2, FRI elf7-2, fri elf8-1, FRI elf8-1, fri efs-3* and *FRI efs-3* as additional controls. (c) Flowering phenotype of *flx-3* and *fll4-1*. All of the expression data are presented as mean values of three biological replicates. Error bars indicate standard deviation.
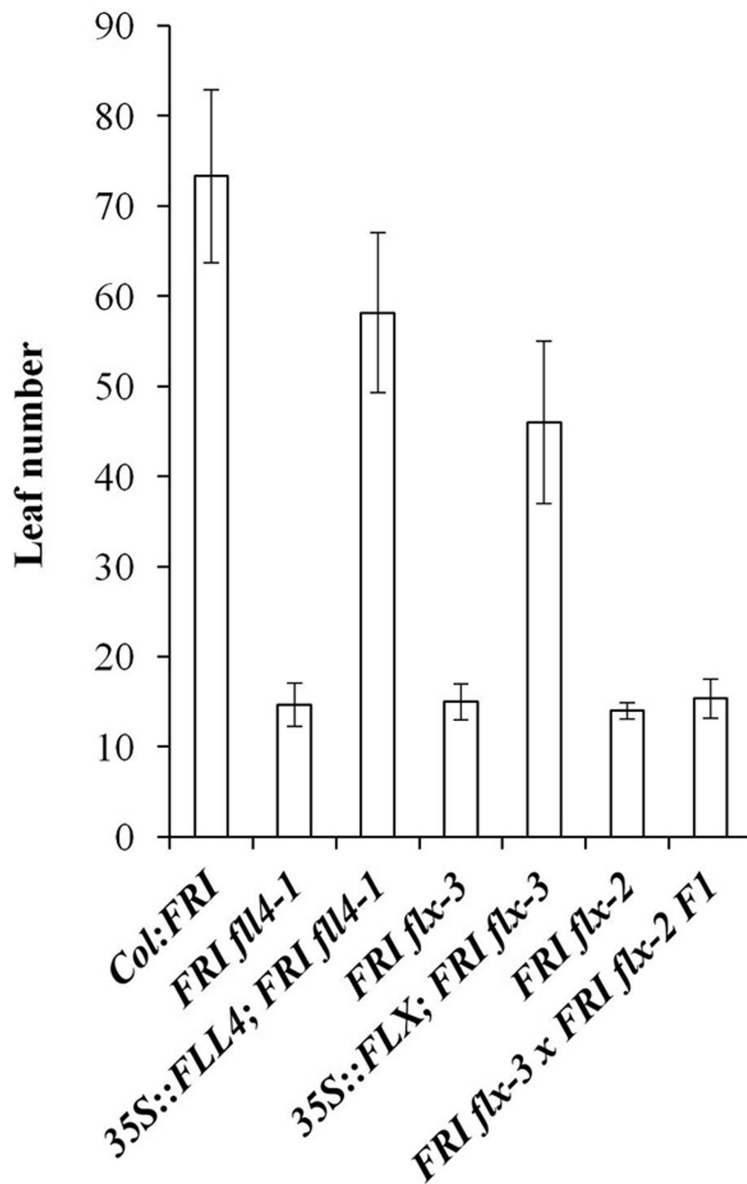
Figure 2. The rapid-flowering phenotype results from lesions in both *FLL4* and *FLX*
Flowering behavior is represented by the number of rosette leaves formed on the primary stem prior to the transition to flowering of plants grown in long days without vernalization. Flowering was monitored in the transgenic lines to determine whether *FLL4* and *FLX* can rescue the flowering phenotype in *FRI fll4* and *FRI flx*. Flowering of *FRI flx-3* × *FRI flx-2* F1 was also monitored. All of the flowering time data are presented as mean values of 12 individual plants except *35S::FLX*; *FRI flx-3* (9 individuals). Error bars indicate standard deviation.

a

b
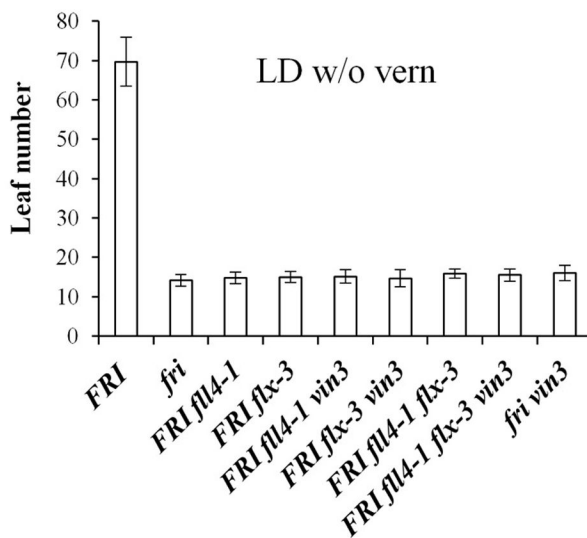
c
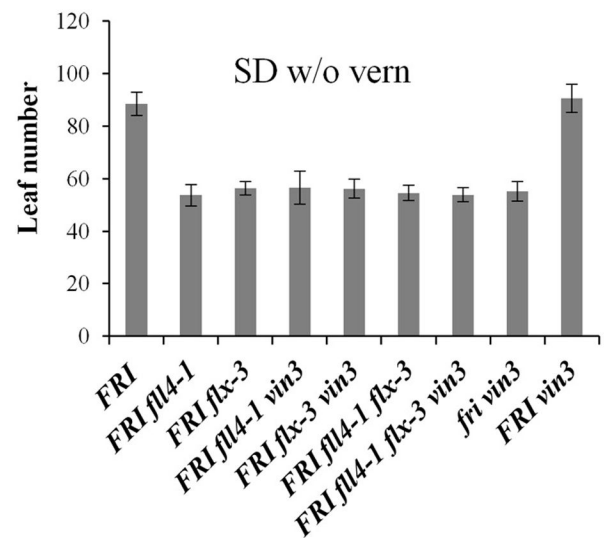
Figure 3. *VIN3* is not required for the rapid flowering of *fll4* and *flx*

The requirement for *VIN3* was evaluated by determining the flowering phenotype of *fll4* and *flx* with and without *VIN3* in long days (a, b) and short days (c). All of the flowering time data are presented as mean values of 12 individual plants. Error bars indicate standard deviation.
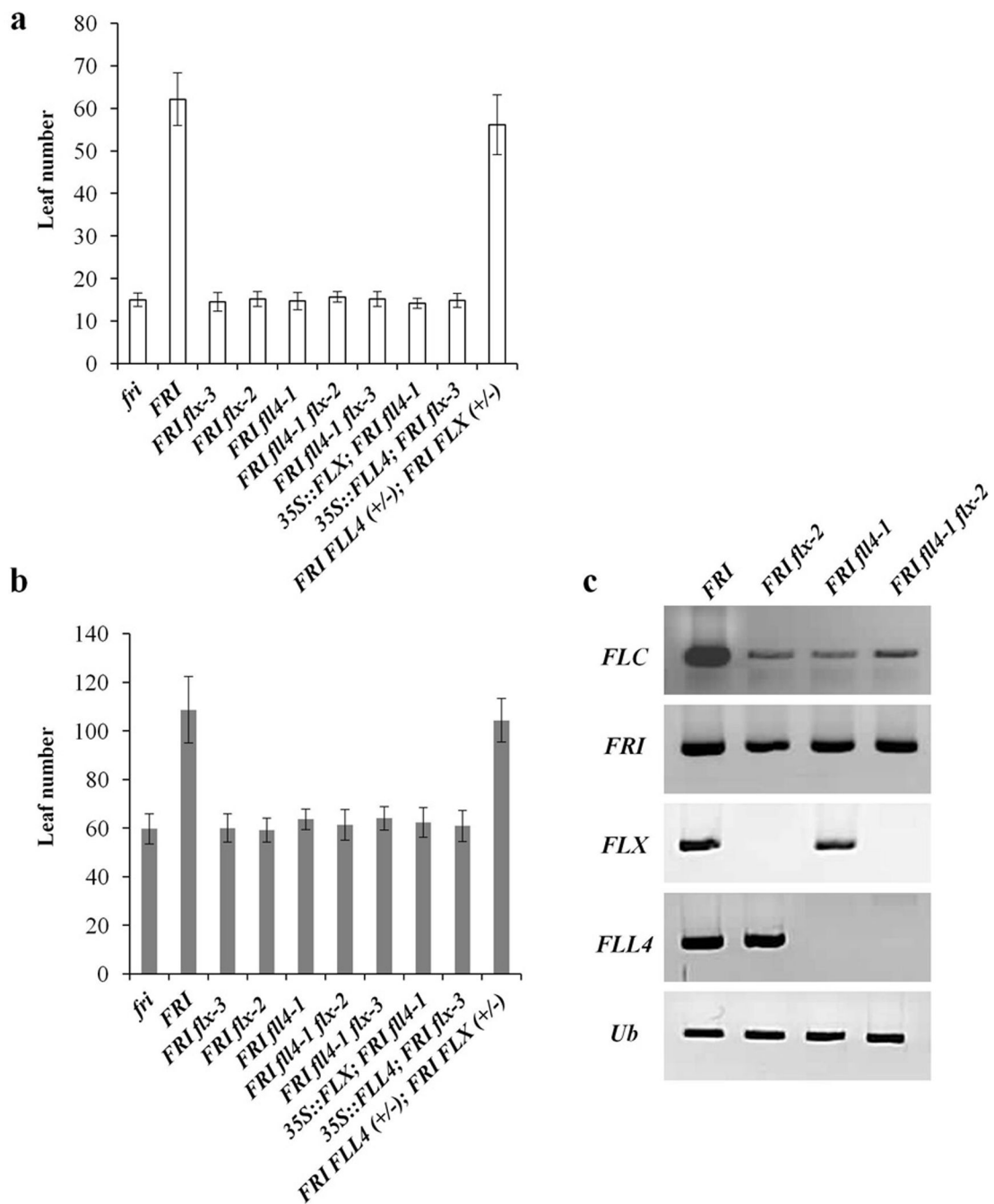
Figure 4. Evaluation of redundancy between *FLL4* and *FLX*

(a) Flowering was monitored in two different versions of the double mutant, *flx-2 fll4-1* and *flx-3 fll4-1* in the presence of *FRI*, relative to each single mutant; the effects of *FLX* overexpression in the *fll4* mutant and *FLL4* overexpression in the *flx* mutant were also determined in long days (a) and short days (b) without vernalization. (c) Transcript levels of *FLC, FRI, FLX* and *FLL4* in the mutant backgrounds were determined. All of the flowering time data are presented as mean values of 12 individual plants. Error bars indicate standard deviation.
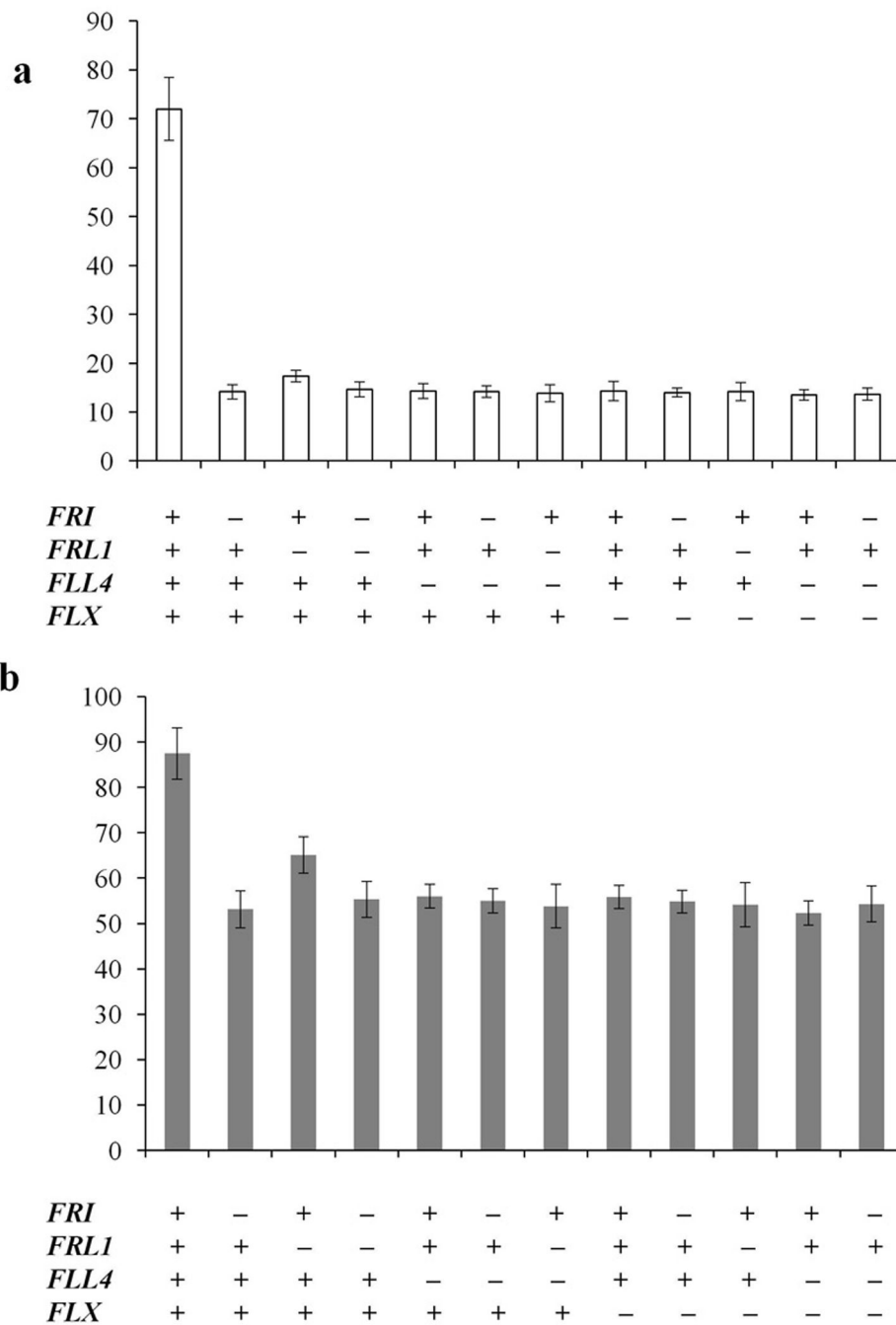
Figure 5. Flowering phenotype of double and triple mutants

Flowering of the indicated mutant combinations was evaluated in long days (a) and short days (b) without vernalization. All of the flowering time data are presented as mean values of 12 individual plants. Error bars indicate standard deviation.
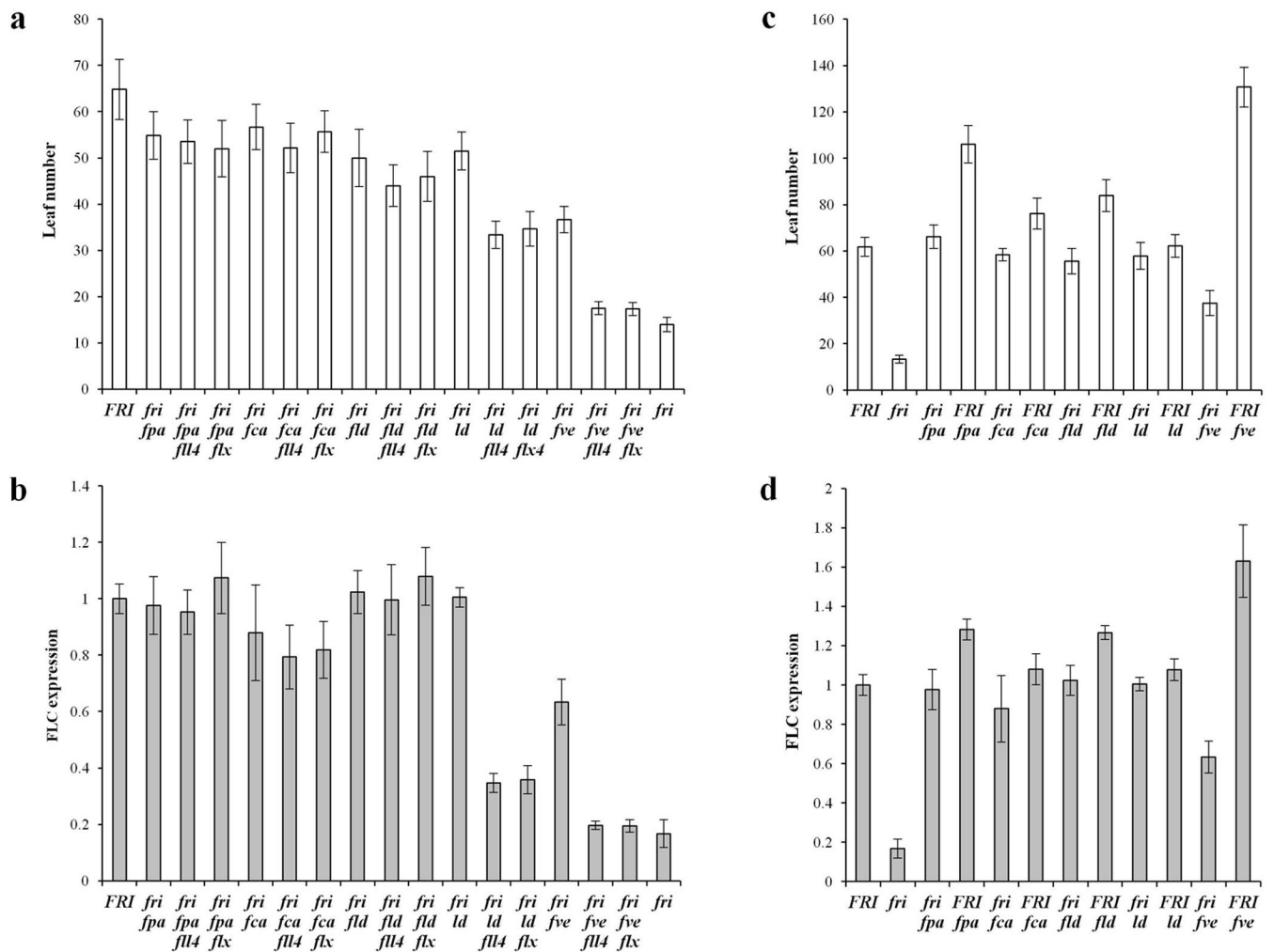
Figure 6. The flowering time and *FLC* expression phenotypes of *fll4* and *flx* combined with other flowering mutants

(a) Flowering of *fll4* and *flx* mutants in autonomous mutants *fpa, fca, fld, ld* and *fve* in long days without vernalization. (b) Transcript levels of *FLC* in the genotypes from (a). (c) Flowering of certain autonomous mutants with or without *FRI*. (d) Transcript levels of *FLC* in the corresponding genotypes from (c). All flowering time data are presented as mean values of 12 individual plants, and all of the expression data are presented as mean values of three biological replicates. Error bars indicate standard deviation.
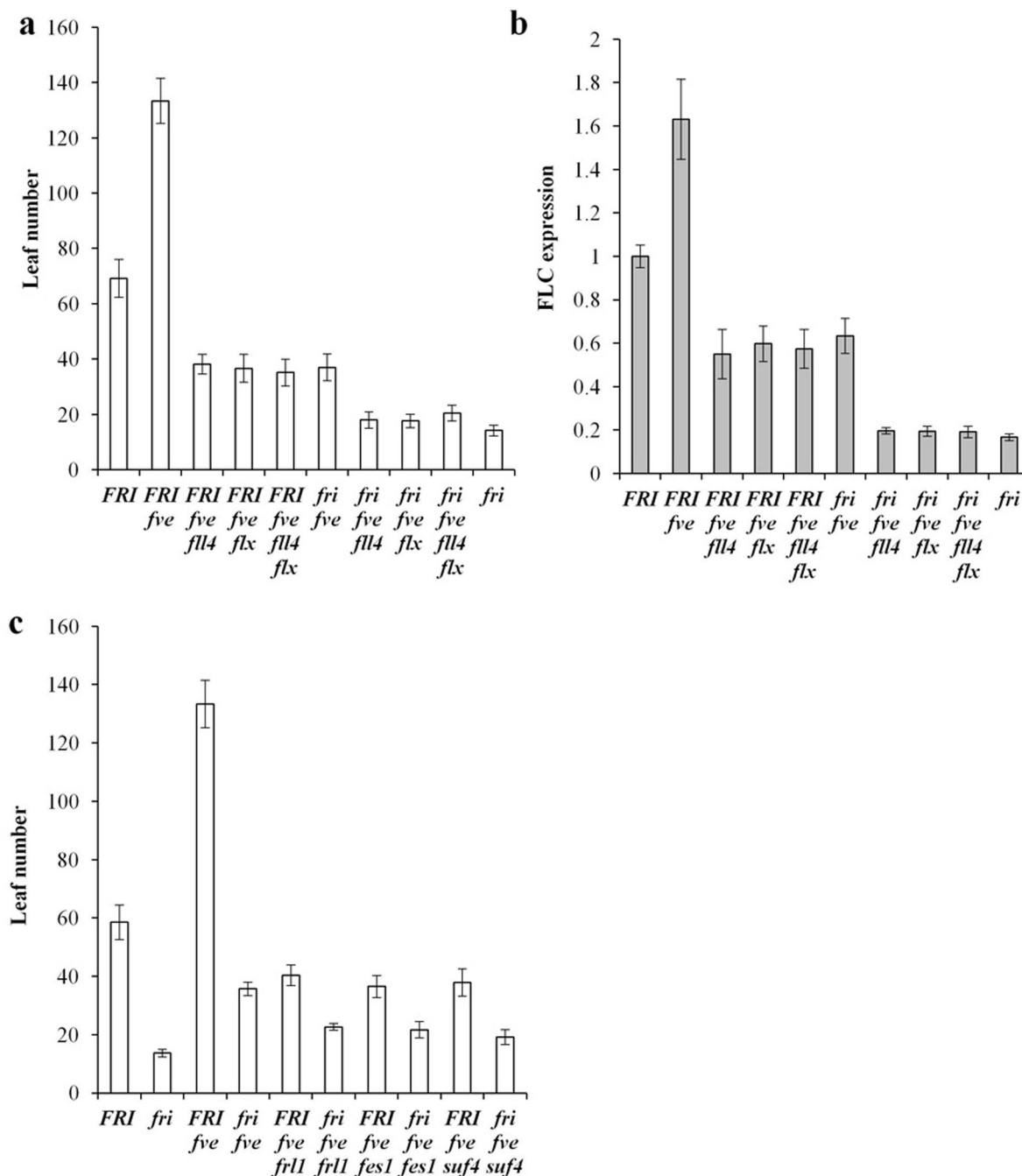
Figure 7. Effect of loss of various *FRI* complex components in the *fve* background
(a) Flowering was evaluated in various mutant combinations of *fri, fll4, flx* and *fve* in long days without vernalization. (b) Transcript levels of *FLC* in the genotypes studied from (a). (c) The flowering phenotype of *FRI* in various FRI complex component mutant backgrounds. All flowering time data are presented as mean values of 12 individual plants, and all of the expression data are presented as mean values of three biological replicates. Error bars indicate standard deviation.