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Ectopic expression of the Arabidopsis florigen gene *FLOWERING LOCUS T* in seeds enhances seed dormancy via the GA and DOG1 pathways

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SUMMARY

Ectopic expression of specific genes in seeds could be a tool for molecular design of crops to alter seed dormancy and germination, thereby improving production. Here, a seed-specific vector, 12S-pLEELA, was applied to study the roles of genes in Arabidopsis seeds. Transgenic lines containing *FLOWERING LOCUS T* (*FT*) driven by the 12S promoter exhibited significantly increased seed dormancy and earlier flowering. Mutated FT(Y85H) and TERMINAL FLOWER1 (TFL1) transgenic lines also showed increased seed dormancy but without altered flowering time. FT(Y85H) and TFL1 caused weaker seed dormancy enhancement compared to FT. The FT and TFL1 transgenic lines showed hypersensitivity to paclobutrazol, but not to abscisic acid in seed germination. The levels of bioactive gibberellin 3 (GA_3) and GA_4 were significantly reduced, consistent with decreased expression of *COPALYL DIPHOSPHATE SYNTHASE* (*CPS*), *KAURENE OXIDASE* (*KO*), *GIBBERELLIN 3-OXIDASE2* (*GA3ox2*), and *GA20ox1* in *p12S::FT* lines. Exogenous GA_{4+7} could recover the germination ability of *FT* transgenic lines. These results revealed that FT regulates GA biosynthesis. A genetic analysis indicated that the GA signaling regulator SPINDLY (SPY) is epistatic to FT in GA-mediated seed germination. Furthermore, *DELAY OF GERMINATION1* (*DOG1*) showed significantly higher transcript levels in *p12S::FT* lines. Seed dormancy analysis of *dog1-2 spy-3 p12S::FT-2* indicated that the combination of SPY and DOG1 is epistatic to FT in the regulation of dormancy. Overall, we showed that ectopic expression of FT and TFL1 in seeds enhances dormancy through affecting GA and DOG1 pathways.

Keywords: seed dormancy, *FLOWERING LOCUS T*, *TERMINAL FLOWER1*, seed-specific expression, gibberellin pathway, *DOG1* pathway.

INTRODUCTION

In many cereal crops, such as wheat (*Triticum aestivum*) and rice (*Oryza sativa*), high temperature and humidity, which frequently happen during late-maturation stages, can cause pre-harvest sprouting and lead to decreased yield. Modification of seed dormancy is considered to be beneficial to prevent pre-harvest sprouting and to improve long-term storage of crop seeds. A full understanding of the molecular mechanisms of seed dormancy would be conducive to the development of gene-modified crops with controllable seed dormancy. This will be beneficial to increase crop productivity and long-term seed storage.

Abscisic acid (ABA) and gibberellin (GA) are considered major phytohormones to control seed dormancy since most of the dormancy-related mutants have defects in ABA and/or GA pathways (Finkelstein *et al.*, 2008; Graeber *et al.*, 2012; Liu and Hou, 2018). ABA is essential for induction and maintenance of seed dormancy (Karssen *et al.*, 1983). Key genes in the ABA pathway, for example, *ABA INSENSITIVE3* and *ABA INSENSITIVE5*, play predominant

roles in seed dormancy regulation (Holdsworth *et al.*, 2008; Koornneef *et al.*, 1984; Kushiro *et al.*, 2004).

Genes involved in GA biosynthesis, for example, *GIBBERELLIN 3-OXIDASE1* (*GA3ox1*), *GA3ox2*, and *GIBBERELLIN 20-OXIDASE2* (*GA20ox2*), promote seed germination by increasing GA content (Mitchum *et al.*, 2006). In contrast, *GIBBERELLIN 2-OXIDASEs* (*GA2oxs*), especially *GA2ox2*, inhibit germination by catalyzing GA deactivation (Yamaguchi, 2008). In the GA signaling pathway, *SPINDLY* (*SPY*) acts as a negative regulator. It encodes an *O*-linked *N*-acetylglucosamine transferase that can modify its target protein by transferring a GlcNac monosaccharide to the *O*-linkage Ser or Thr to modulate their activities (Qin *et al.*, 2011; Shimada *et al.*, 2006). The *spy* mutants exhibit decreased dormancy and show resistance to the GA biosynthesis inhibitor paclobutrazol (PAC) (Jacobsen and Olszewski, 1993; Swain *et al.*, 2001). DELLA proteins are well-known negative regulators of GA signal transduction and are involved in all aspects of GA-induced development (Tyler *et al.*, 2004). Among them, RGA-LIKE2 (RGL2) plays a predominant role in seed dormancy regulation. Loss-of-function mutants of *RGL2* show decreased dormancy and substantial resistance to PAC (Lee *et al.*, 2002; Peng and Harberd, 2002).

Recent work has indicated that flowering and seed dormancy, two key events in the plant life cycle, are not controlled independently. Some central regulatory factors in the flowering pathway were found to be associated with seed dormancy, such as *FLOWERING LOCUS C* and *FY* (Chiang *et al.*, 2009; Hughes *et al.*, 2019; Jiang *et al.*, 2012). This may be advantageous for plants by adjusting the timing of germination with the environmental conditions encountered during flowering. *FLOWERING LOCUS T* (*FT*), which has a central role in flowering time control, acts as a mobile signal (Corbesier *et al.*, 2007; Jaeger and Wigge, 2007). It integrates signals from the photoperiod, vernalization, autonomous, and GA pathways in leaves, and then travels in the phloem to transmit the signal to the shoot apex (Taoka *et al.*, 2013). Recent work showed that FT inhibits seed dormancy in Arabidopsis by maternally regulating phosphatidic acid synthesis in the seed coat of seeds with a Landsberg *erecta* (L*er*) background, but not in that of seeds with a Columbia (Col) background (Chen *et al.*, 2014; Chen and Penfield, 2018). FT, as well as TERMINAL FLOWER1 (TFL1) and MOTHER OF FT and TFL1 (MFT), belongs to the phosphatidylethanolamine-binding protein (PEBP) family with unknown molecular function (Karlgren *et al.*, 2011). With high similarity in protein sequence to FT, TFL1 plays an opposite role to FT in regulating flowering time (Wickland and Hanzawa, 2015). MFT predominantly participates in regulating seed germination (Vaistij *et al.*, 2018; Xi and Yu, 2010).

Many of the genes that have been reported to function in seed dormancy regulation have multiple functions (Finkelstein and Somerville, 1990; Koornneef and van der Veen, 1980; Leon-Kloosterziel *et al.*, 1996). Only a few seed-specific genes have been identified as exclusive dormancy regulators. *DELAY OF GERMINATION1* (*DOG1*), belonging to a small gene family with unknown molecular function, has been identified to be a regulator underlying natural variation of seed dormancy (Alonso-Blanco *et al.*, 2003; Bentsink *et al.*, 2006, 2010). *REDUCED DORMANCY5* (*RDO5*) is a dormancy regulator isolated by mapping of non-dormant *rdo5*, a γ-irradiation-induced mutant in a NIL-DOG1 background (Xiang *et al.*, 2014). RDO5 is a PP2C pseudophosphatase and interacts with DOG1, indicating that the genetic relationship between DOG1 and RDO5 is associated with protein interaction (Née *et al.*, 2017; Xiang *et al.*, 2016).

In agriculture, it is preferred to directly modify certain traits without influencing other aspects of plant growth and development. Therefore, tissue-specific promoters are promising choices. In the present study, a seed-specific expression system was exploited to express *FT* in seeds to investigate its role in seed dormancy regulation. The information obtained from this study contributes to our understanding of the molecular mechanisms of seed dormancy and provides tools for the development of crop variants with properly controlled seed dormancy.

RESULTS

Ectopic expression of *FT* in seeds causes enhanced seed dormancy

To specifically investigate the roles of pleiotropic genes in seed dormancy, a 12S promoter, from *At4g28520* encoding a 12S storage protein, was cloned and used to construct a seed-specific vector, named 12S-pLEELA. To test the expression and tissue specificity of this vector in the Col background, we inserted the β-glucuronidase (GUS)-encoding gene (*uidA*), resulting in the p12S::GUS construct. The p35S::GUS construct was used as a control. GUS staining analysis of independent *p12S::GUS* transgenic lines showed an initial signal in the embryo at the 7th day after flowering (daf). The signal gradually increased in the embryo along seed maturation and decreased during seed dehydration (Figure S1a,g). The GUS signal was detected in cotyledons, hypocotyl, and axillary buds after germination; the signal was undetectable in other tissues (Figure S1b–f). Quantitative PCR (Q-PCR) analysis showed that the relative expression level of *uidA* driven by the 12S promoter varied dramatically between different *p12S::GUS* lines, and was on average much higher compared to *uidA* expression driven by the 35S promoter (Figure S1g). This indicated that it is possible to obtain transgenic lines with diverse expression levels of target genes in seeds by using 12S-pLEELA.

The *FT* gene was subsequently cloned into 12S-pLEELA, and independent homozygous single-copy transgenic

plants in the Col background were obtained. Interestingly, compared with Col, the *p12S::FT* transgenic lines exhibited enhanced seed dormancy to different degrees (Figure 1a, b). Among those lines, *p12S::FT-6* displayed the most significant phenotype with deep dormancy remaining after 12-week dry storage (Figure S2a). Q-PCR analysis showed different expression levels of *FT* in each *p12S::FT* line, and the highest expression level was observed in *p12S::FT-6* (Figure 1c). Accordingly, the FT protein levels in dry *p12S::FT-6* seeds were much higher than in *p12S::FT-2* (Figure 1d). The dormancy level of each line was generally correlated with the relative expression levels of *FT* in their seeds, indicating that the enhancement of seed dormancy by *FT* is dose-dependent.

Four *p12S::FT* transgenic lines with high *FT* expression levels in seeds showed earlier flowering time compared with Col (Figure S2b–d). As a result of differences in flowering time, seed development and maturation could happen at different times for Col and *p12S::FT* lines. Therefore, the seed dormancy phenotypes of *p12S::FT* transgenic

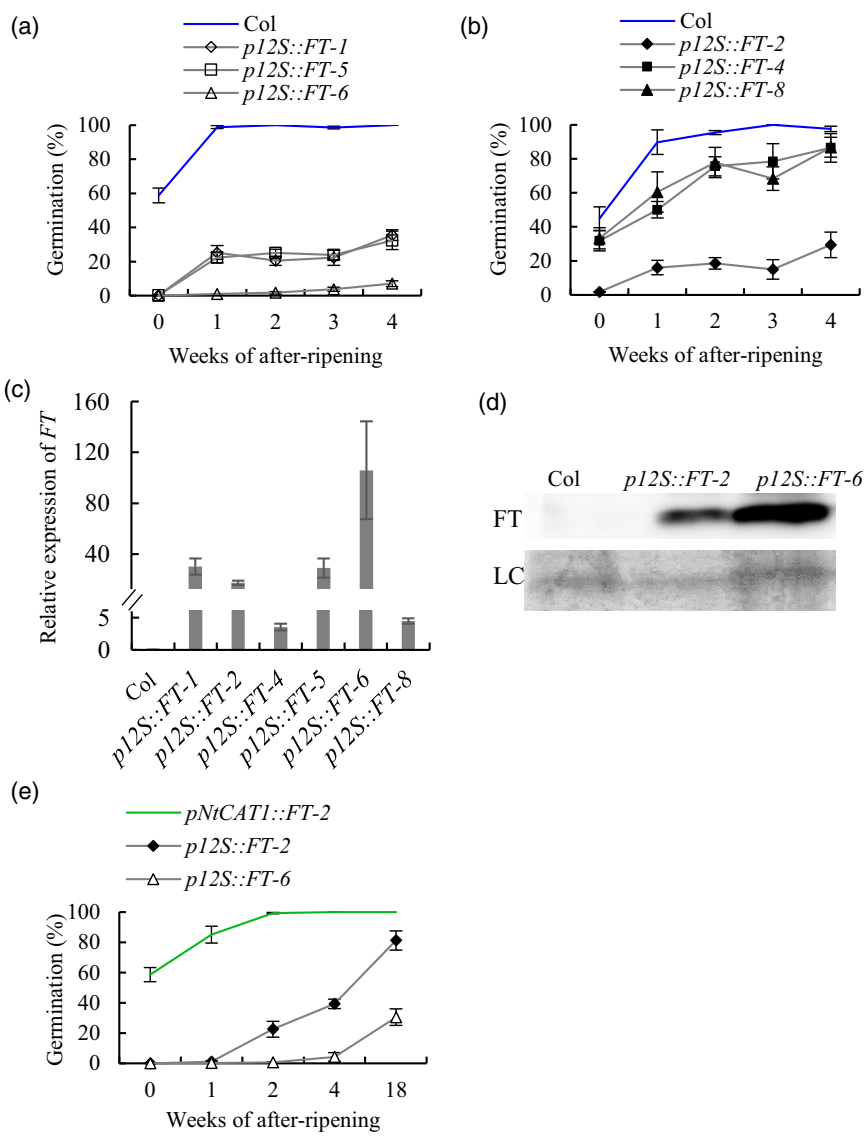

Figure 1. The *p12S::FT* transgenic lines show enhanced seed dormancy.
(a, b) Germination percentages of Col and *p12S::FT* transgenic lines at different moments post-ripening. Percentages are averages (±SE) based on eight individual plants for each line. (c) Quantification of relative expression levels of *FLOWERING LOCUS T* (*FT*) in freshly harvested seeds of Col and *p12S::FT* transgenic lines by Q-PCR. *ACTIN8* was used as an internal control. Values are averages (±SE) from three biological replicates. (d) Immunoblot analysis of FT protein accumulation in dry seeds of *p12S::FT* transgenic lines. Coomassie blue staining was used as loading control as shown in the picture below. 'LC' indicates 'loading control'. (e) Germination percentages of *pNtCAT1::FT* line 2, *p12S::FT* line 2, and *p12S::FT* line 6 at different moments post-ripening. Germination percentages are averages (±SE) based on 8–10 individual plants for each line.

lines could be influenced by different environmental conditions during seed development and maturation. To eliminate the possible effect of earlier flowering on seed dormancy of *p12S::FT* lines, we constructed a *pNtCAT1::FT* homozygous line, namely *pNtCAT1::FT-2*, as an additional control. The *NtCAT1* promoter was used as an endosperm-specific promoter in a previous study (Nakabayashi *et al.*, 2012). As shown in Figure S2(e), the expression levels of *FT* driven by the *NtCAT1* promoter in dry seeds were significantly lower than in *p12S::FT-4* seeds. *pNtCAT1::FT-2* showed no difference in dormancy level with Col (Figure S2g), but exhibited earlier flowering, similar to strong *p12S::FT* transgenic lines (Figure S2f). Germination tests indicated that the *p12S::FT* transgenic lines were much more dormant than *pNtCAT1::FT-2* (Figure 1e). These results suggested that the enhanced dormancy of *p12S::FT* transgenic lines was not caused by environmental variation during seed maturation due to differences in flowering time.

Moreover, two *p12S::GUS* lines with different *uidA* expression levels in seed were introduced as negative controls (Figure S2h). Both lines showed no difference in seed dormancy compared with Col (Figure S2i). This indicated that the dormancy phenotypes of *p12S::FT* transgenic lines were not caused by a transgene containing a 12S promoter, but by the property of the *FT* gene.

Lower germination of *p12S::FT* transgenic lines is not due to seed viability

It could be possible that the lower seed germination of *p12S::FT* transgenic lines does not result from dormancy, but from decreased seed viability. Therefore, the *p12S::FT-2* and *p12S::FT-6* lines with moderate and high dormancy levels were analyzed in several seed viability experiments (Figure 2). The 2,3,5-triphenyltetrazolium chloride (TTC) assay, which is a classical method to examine seed viability, showed no substantial difference in seed color between *p12S::FT* transgenic lines and Col when treated for either 60 h or 1 week in TTC buffer in the dark (Figure 2a). At this point, *p12S::FT* transgenic lines still showed significantly lower germination (Figure 2a). Moreover, by peeling off seed coats and endosperms, the isolated embryos of *p12S::FT* seeds reached 100% germination, indicating that they were fully viable. By contrast, the intact seeds still showed significantly lower germination, which was about 33% for *p12S::FT-2* and 1.8% for *p12::FT-6* (Figure 2b; Figure S2j). Finally, as low temperatures can release seed dormancy in Arabidopsis, germination tests after stratification (imbibition at 4°C for 3–4 days) were carried out. The results showed that stratification could rescue seed germination to 100% for *p12S::FT-2* and 80% for *p12S::FT-6* (Figure 2c). Altogether, we can conclude that the lower germination of *p12S::FT* transgenic lines is caused by enhanced seed dormancy and not by decreased seed viability.

Tyr85 of the FT protein is not essential for modification of seed dormancy

A previous study indicated that the Y85H substitution of FT causes its functional conversion to TFL1-like in flowering time control (Hanzawa *et al.*, 2005). Using site-directed mutagenesis, we constructed the *p12S::FT(Y85H)* plasmid and obtained five independent homozygous transgenic lines. The *p12S::FT*(*Y85H*) transgenic lines exhibited different degrees of enhanced seed dormancy compared with Col. The dormancy level of each line was in general correlated with their respective expression level of *FT*(*Y85H*) in seeds (Figure 3a–d). Compared with *p12S::FT-2*, *p12S::FT*(*Y85H*)*-12* displayed similar expression levels but much weaker dormancy (Figure 3c,d). This was also observed in the PAC sensitivity test (Figure 5d). The *p12S::FT*(*Y85H*)*-12* line showed less sensitivity in response to PAC than *p12S::FT-2*. These results indicated that the Y85H mutation weakened the function of FT in the regulation of seed dormancy, but did not abolish it.

In contrast to *p12S::FT* transgenic lines, the *p12S::FT*(*Y85H*) lines did not differ in flowering time compared with Col (Figure S3a–c). Therefore, Tyr85 is important for the full function of FT in enhancing seed dormancy and flowering time regulation, but not essential for FT in regulating seed dormancy.

High expression of *TFL1* in seeds enhances dormancy

TFL1 is closely related to FT in protein sequence and three-dimensional structure, but plays an opposite role in regulating flowering time in Arabidopsis (Wickland and Hanzawa, 2015). We cloned the *TFL1* gene to investigate its role in seed dormancy. Four *p12S::TFL1* independent homozygous lines were obtained. Germination tests showed that the *p12S::TFL1* transgenic lines had different levels of enhanced dormancy, which was generally correlated with their *TFL1* expression level in seeds (Figure 4a, b). We noticed that *TFL1* requires higher expression levels than *FT* to obtain similar dormancy levels (Figure 4b,c). Finally, all *p12S::TFL1* transgenic lines exhibited similar flowering times as the Col wild type (Figure S3d). These results indicate that *TFL1* has a dose-dependent enhancing effect seed dormancy, but with weaker efficiency than *FT*.

FT, *TFL1*, and mutated *FT(Y85H)* transgenic lines showed hypersensitivity to PAC but not to ABA

As GA and ABA are key hormones in controlling seed dormancy, their seed germination sensitivity was analyzed to examine whether these hormones are involved in *FT*-regulated seed dormancy and germination. The application of different concentrations of PAC, an inhibitor of GA biosynthesis, during imbibition showed hypersensitivity in seed germination of both *p12S::FT-2* and *p12S::FT-6* compared with Col (Figure 5a). This inhibition by PAC was

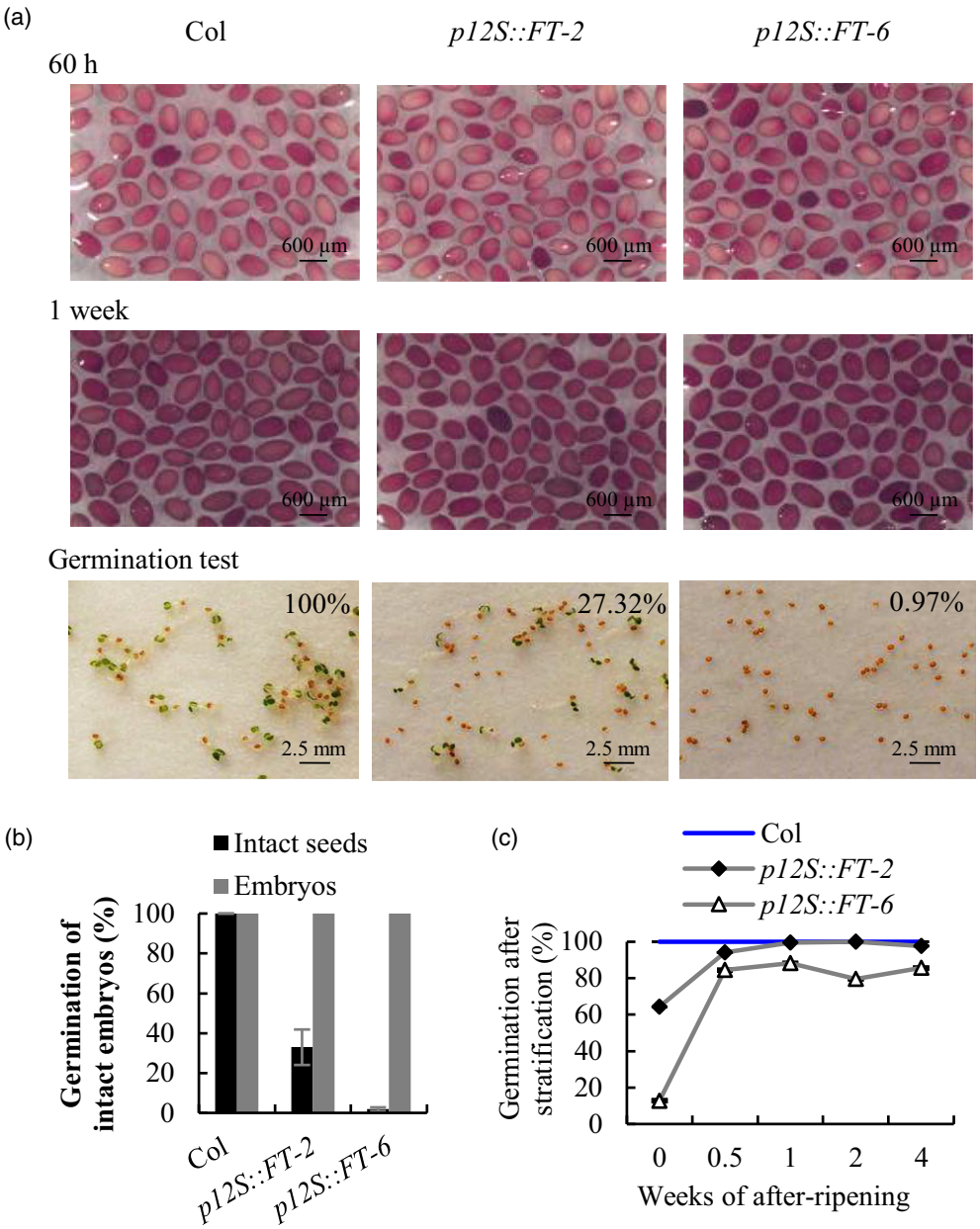

Figure 2. Col and *p12S::FT* transgenic lines have a similar seed viability.
(a) Staining after a 2,3,5-triphenyltetrazolium chloride (TTC) assay. The upper photos represent seeds of Col and *p12S::FT* transgenic lines treated for 60 h in TTC buffer. The middle photos show seeds of Col and *p12S::FT* transgenic lines treated for 1 week in TTC buffer. The bottom photos show germination of Col and *p12S::FT* transgenic lines after 7-day imbibition. The germination percentages are indicated in the photo. (b) Germination of isolated embryos and intact seeds. Germination percentages are averages (±SE) based on eight individual plants for each line. (c) Germination percentages of Col and *p12S::FT* transgenic lines after stratification and different storage periods. Germination percentages are averages (±SE) based on six to eight individual plants for each line.

completely rescued in the presence of 10 μM GA_{4+7} (Figure 5b). Moreover, seed dormancy of both *p12S::FT-2* and *p12S::FT-6* could be completely released by adding 21 μM GA_{4+7} without stratification (Figure 5c). These results indicate that FT might be involved in the GA pathway to regulate seed dormancy and germination.

Additionally, *p12S::FT*(*Y85H*) and *p12S::TFL1* lines also showed hypersensitivity to PAC (Figure 5d,e), indicating that the dormancy phenotypes of these transgenic lines are also associated with the GA pathway.

In response to exogenous ABA, most of the *p12S::FT* lines did not show changes in seed germination compared to the Col wild type, even when treated with high levels of ABA. Only the most dormant line, *p12S::FT-6*, showed slightly enhanced sensitivity (Figure 5f; Figure S4a). In addition, the *p12S::FT*(*Y85H*) and *p12S::TFL1* lines showed

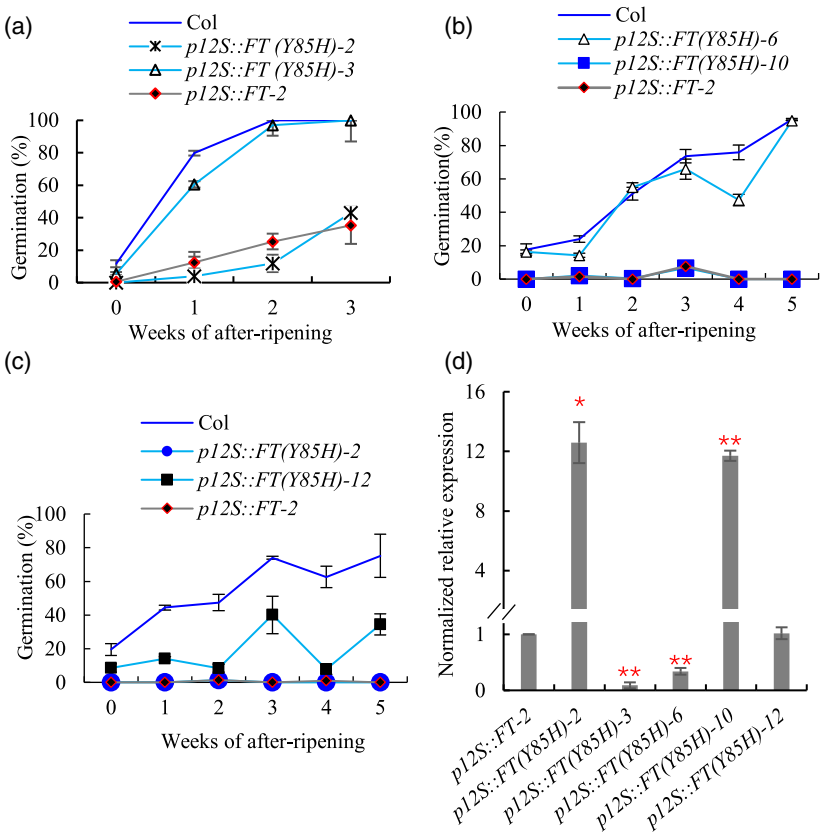

Figure 3. The *p12S::FT(Y85H)* transgenic lines show enhanced seed dormancy.
(a–c) Germination percentages of different *p12S::FT (Y85H)* transgenic lines at different moments post-ripening. Percentages are averages (±SE) based on eight individual plants for each line. (d) Quantification of relative expression by Q-PCR of *FT(Y85H)*/*FT* in transgenic lines. The same primers were used for both *FT(Y85H)* and *FT*. Values of *FT(Y85H)* in *p12S::FT(Y85H)* lines were normalized to *FT* in *p12S::FT-2*. *ACTIN8* was used as an internal control. Values are averages (±SE) from three biological replicates. Significant differences between *p12S::FT(Y85H)* transgenic lines and *p12S::FT-2* were determined using Student's *t*-test (*$P < 0.05$, **$P < 0.01$).

similar or slightly altered seed germination compared to Col in response to ABA (Figure S4b,c). These results suggest that *FT*-regulated seed dormancy does not, or only slightly, involve the ABA pathway.

FT influences the GA biosynthesis and signaling pathways

We were interested whether *FT* expression in seeds influences the transcript levels of genes involved in GA biosynthesis. Therefore, the expression levels of critical genes involved in the GA pathway were analyzed by Q-PCR using seeds isolated from siliques at different maturation stages or fresh seeds imbibed for 0, 6, 12, or 24 h. As shown in Figure 6(a), the expression levels of *CPS* and *KO*, which are involved in early GA biosynthesis stages (Hedden and Thomas, 2012), were significantly decreased in the *p12S::FT-6* line during seed maturation and imbibition, and a relative slight decrease was also observed in the *p12S::FT-2* line. The final two steps in GA biosynthesis are conducted by GA20oxs and GA3oxs, among which *GA20ox1*, *GA20ox2*, *GA3ox1*, and *GA3ox2* have relatively predominant roles in seed dormancy and germination (Ogawa *et al.*, 2003). The expression of *GA20ox1* was significantly reduced in *p12S::FT* lines during most stages. The expression of *GA3ox2* in *p12S::FT-6* was significantly decreased during imbibition. *GA20ox2* and *GA3ox1* also showed decreased expression although this decrease is not statistically significant in most cases. Overall, these results indicate that *FT* plays a negative role in the GA biosynthesis pathway in a dose-dependent manner. Consistent with the decreased expression levels of genes involved in GA biosynthesis, contents of bioactive GA_1, GA_3, GA_4, and GA_7 were all lower in *p12S::FT-6* seeds imbibed for 24 h compared to Col wildtype, which was significant for GA_3 and GA_4 (Figure 6c). Finally, in accordance with decreased GA biosynthesis, some GA-related genes involved in cell wall remodeling during seed germination, for example, genes encoding group A EXPANSINS (EXPs) and XYLOGLUCAN ENDOTRANS-GLUCOSYLASE/HYDROLASES (XTHs), were also expressed at significantly lower levels in the *p12S::FT* transgenic lines compared to Col during seed imbibition (Figure S5). These results suggest that *FT* regulates seed dormancy and germination by inhibiting GA biosynthesis.

To gain more insight in the relation between FT and the GA pathway, we performed genetic analyses. SPY and RGL2 are two negative factors in GA signaling with positive roles in seed dormancy (Jacobsen *et al.*, 1996; Lee *et al.*, 2002). We constructed *spy-3 p12S::FT-2* and *rgl2-13 p12S::FT-2* homozygous plants, and analyzed their seed dormancy phenotypes and PAC sensitivity during seed germination. Both *spy-3 p12S::FT-2* and *rgl2 p12S::FT-2* showed partial rescue of the *p12S::FT-2* seed dormancy

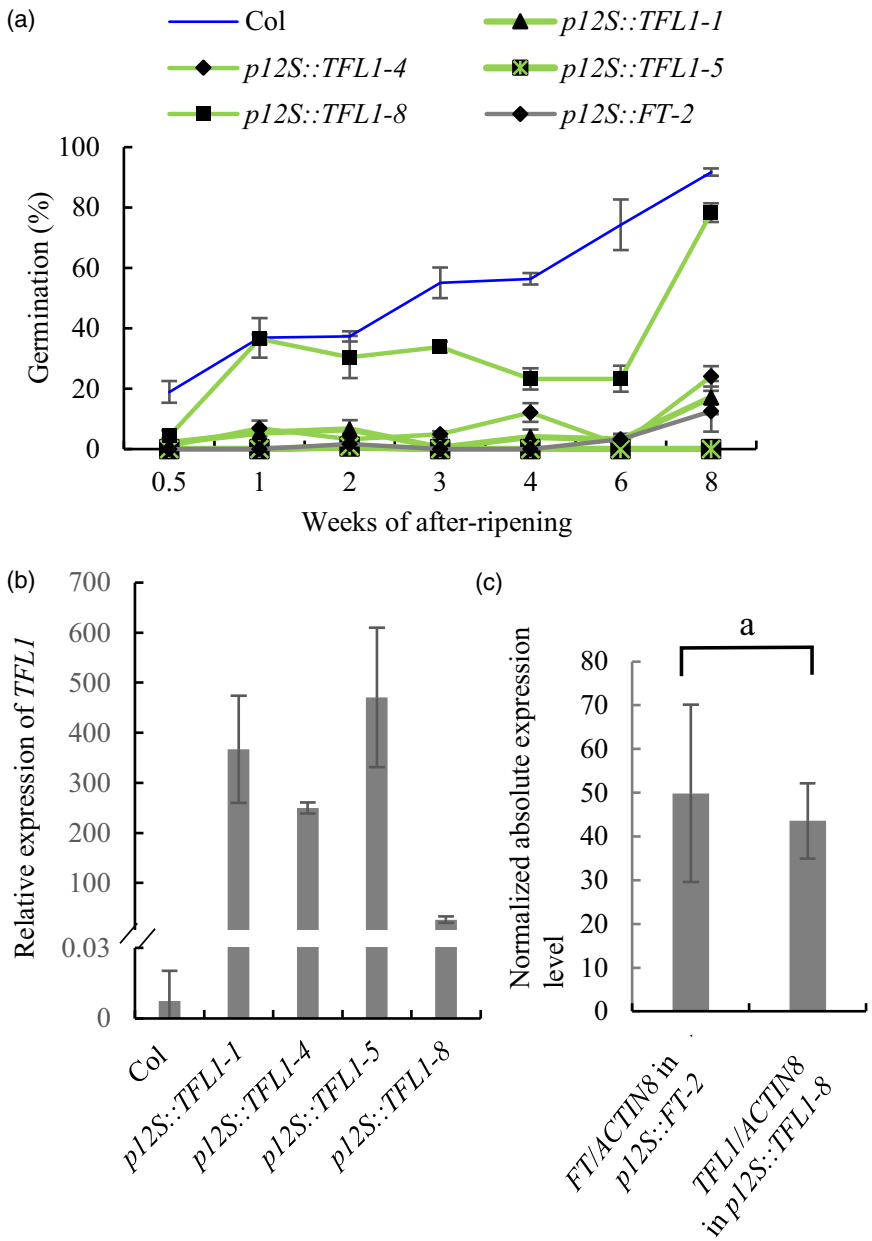

Figure 4. The *p12S::TFL1* transgenic lines show enhanced seed dormancy.
(a) Germination percentages of Col and *p12S::TFL1* transgenic lines at different moments post-ripening. Percentages are averages (±SE) based on six to eight individual plants for each line. (b) Quantification of the relative expression of *TFL1* in freshly harvested seeds of the transgenic lines by Q-PCR. *ACTIN8* was used as an internal control. Values are averages (±SE) from three biological replicates. (c) Quantification of the absolute expression of *TFL1* in dry seeds of *p12S::TFL1-8* and *FT* in dry seeds of *p12S::FT-2* by Q-PCR. Absolute expression of *ACTIN8* in these lines was used as an internal control to normalize cDNA concentrations. Values are averages (±SE) from three biological replicates. 'a' indicates no significant difference as determined by Student's *t*-test ($P < 0.05$).

phenotype (Figure 7a; Figure S6a). However, seed germination sensitivity to PAC of *spy-3 p12S::FT-2* was identical to that of *spy-3* (Figure 7b), indicating that SPY is epistatic to FT in GA-regulated seed germination. RGL2 is not completely epistatic to FT. Although *rgl2-13 p12S::FT-2* shows strongly reduced PAC sensitivity compared to *p12S::FT-2*, it is significantly more sensitive than *rgl2-13* when applied with high concentrations of PAC (Figure S6b).

DOG1 is involved in *FT*-regulated primary seed dormancy

DOG1 is an important factor in seed dormancy regulation, which promotes primary seed dormancy establishment during seed maturation. We examined *DOG1* expression levels during seed maturation and imbibition and found a higher abundance during all stages in *p12S::FT-2* compared to the Col wild type (Figure 6b). Germination tests of

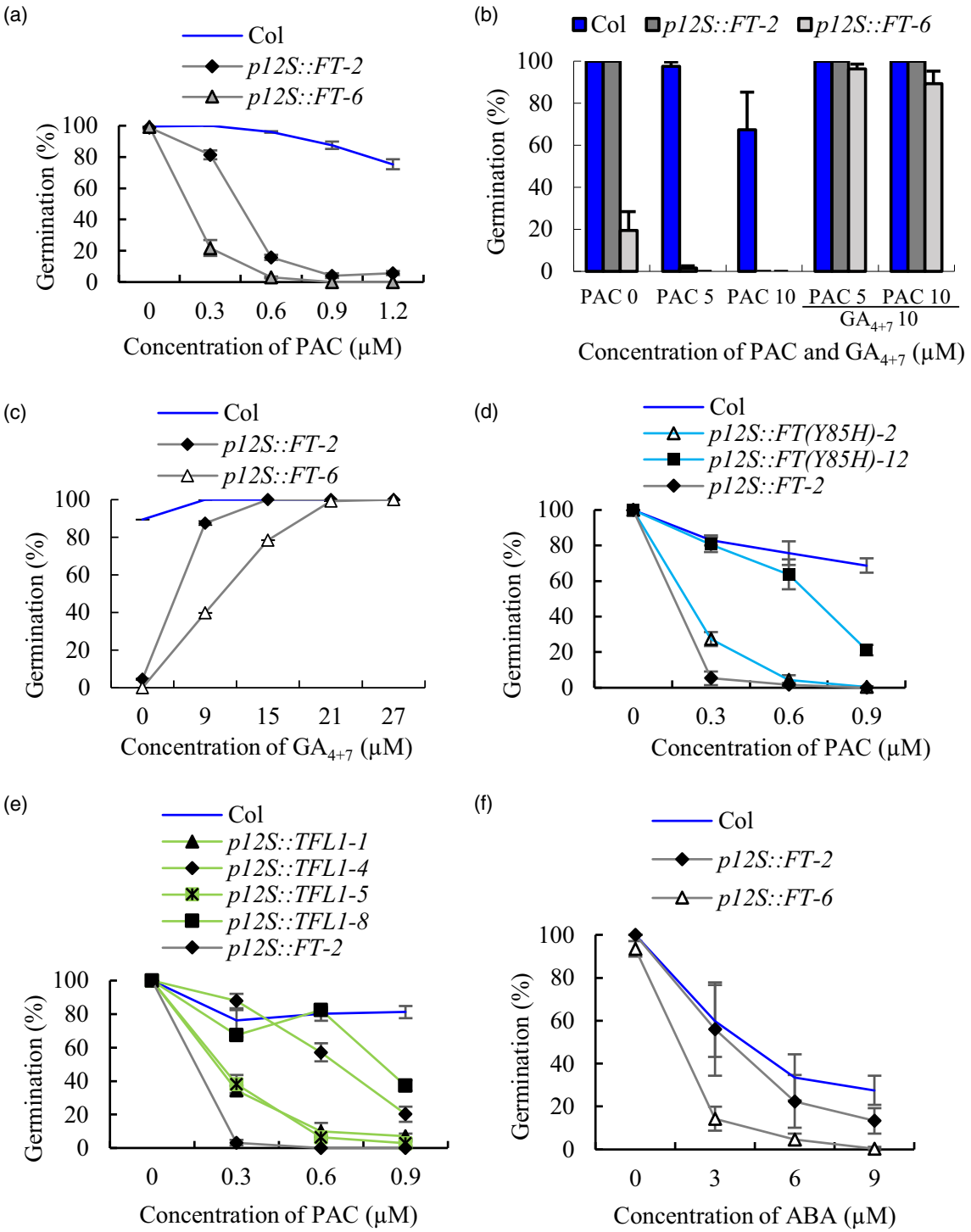

Figure 5. Ectopic expression of *FLOWERING LOCUS T (FT), FT(Y85H),* and *TFL1* in seeds affects the sensitivity of seed germination to paclobutrazol (PAC). (a) Seed germination of Col and *p12S::FT* transgenic lines in response to different concentrations of PAC. (b) Seed germination of Col and *p12S::FT* transgenic lines in response to different concentrations of PAC or PAC coupled with 10 μM GA_{4+7}. (c) Seed germination of Col and *p12S::FT* transgenic seeds treated with different concentrations of GA_{4+7} without stratification. (d). Seed germination of Col and *p12S::FT(Y85H)* transgenic lines in response to different concentrations of PAC. (e) Seed germination of Col and *p12S::TFL1* transgenic lines in response to different concentrations of PAC. (f) Seed germination of Col and *p12S::FT* transgenic lines in response to different concentrations of abscisic acid (ABA). Percentages are averages (±SE) based on six to eight individual plants for each line.

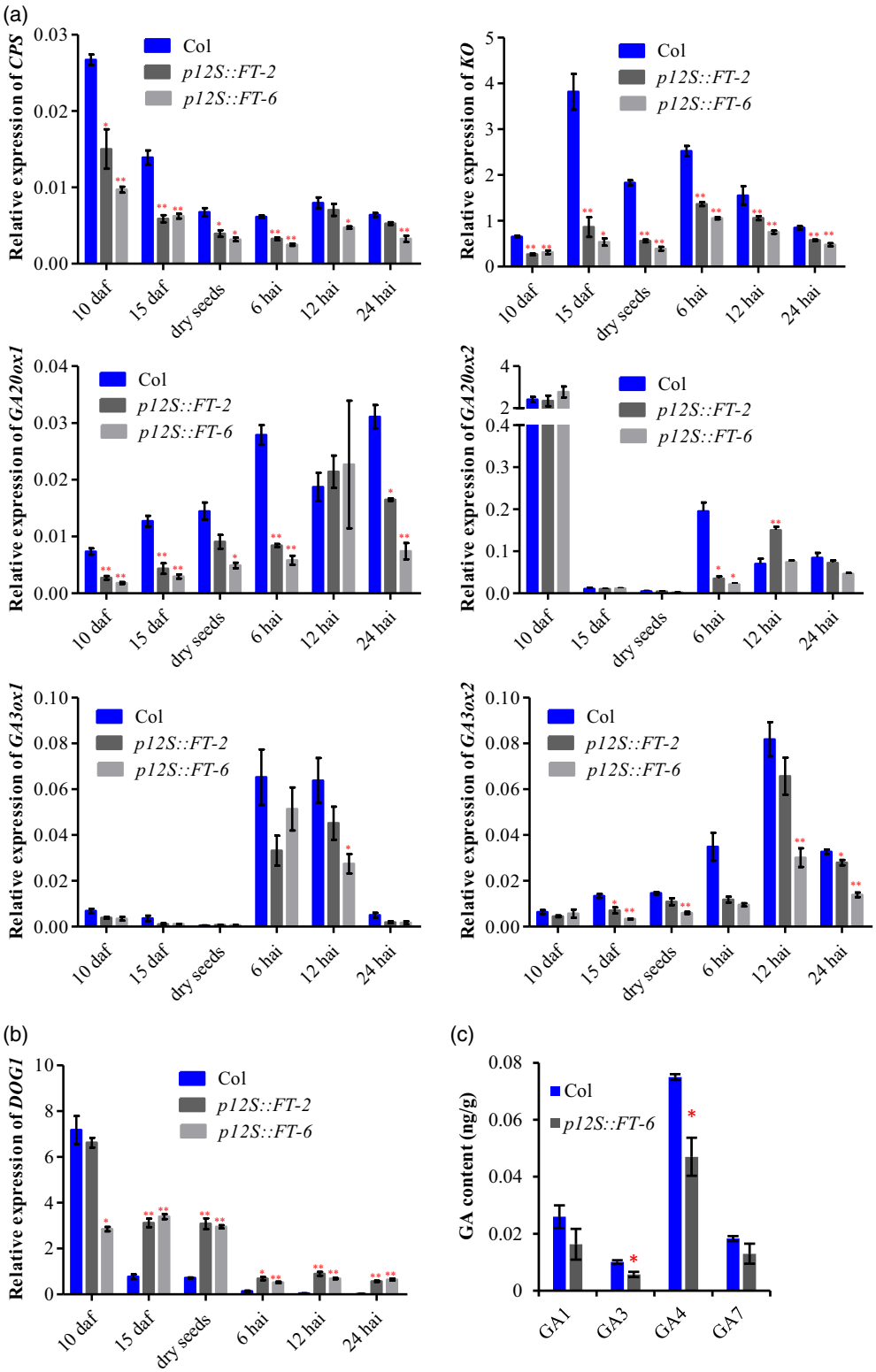

Figure 6. *FLOWERING LOCUS T* (*FT*) reduces transcript levels of gibberellin (GA) biosynthesis-related genes and GA contents in seeds, but enhances *DOG1* transcript levels.

(a, b) Relative expression levels of *CPS*, *KO*, *GA3ox1*, *GA3ox2*, *GA20ox1*, *GA20ox2*, and *DOG1* were examined by Q-PCR. RNA was extracted from seeds isolated from siliques grown for 10 or 15 days after flowering (daf) and from freshly harvested seeds imbibed for 0, 6, 12, or 24 h. *ACTIN8* was used as an internal control. Values are averages (±SE) based on three biological replicates. Significant differences between *p12S::FT* transgenic lines and wild type were determined using Student's *t*-test (**P* < 0.05, ***P* < 0.01). (c) Biochemical analysis of GA contents in Col and *p12S::FT-6* seeds imbibed for 24 h. Values are averages (±SE) from three biological replicates. Significant differences compared with the wild type were determined using Student's *t*-test (**P* < 0.05, ***P* < 0.01).

dog1-2 p12S::FT-2 showed that its dormancy level is in between those of *dog1-2* and *p12S::FT-2* (Figure 7c), indicating that DOG1 is not the only factor through which FT induces dormancy. Thus, the GA and DOG1 pathways were both found to be involved in FT-mediated seed dormancy and germination. To further dissect the genetic relationship between *FT*, *DOG1*, and *SPY*, we combined *dog1-2*, *spy-3*, and *p12S::FT-2* in the same background (Figure 7d). An analysis of germination showed that freshly harvested seeds of *dog1-2 spy-3 p12S::FT-2* are completely non-dormant (Figure 7d). Therefore, FT is not able to induce dormancy in a genetic background with inactive DOG1 and a constitutively activated GA pathway.

DISCUSSION

As seed dormancy is a quantitative trait influenced by environmental factors during maternal development and seed storage, it is difficult to compare seed dormancy levels between experiments, even with accurately controlled environmental conditions (Donohue *et al.*, 2008; Fenner, 1991; Schmuths *et al.*, 2006). Evaluation of seed dormancy also becomes more difficult when mutants influence developmental traits, such as flowering time. To explore the specific function of genes in seed dormancy, we constructed a vector with seed-specific expression, namely 12S-pLEELA. Analysis of transgenic lines containing the GUS-encoding gene driven by the 12S promoter showed GUS signals in embryos during seed development and weak signals in cotyledons and hypocotyl after germination (Figure S1a–f). Q-PCR analysis indicated that the average *uidA* mRNA expression level in *p12S::GUS* seeds was often much higher than that in *p35S::GUS* seeds (Figure S1g). This indicates that the 12S-pLEELA vector is a useful tool to obtain a high level of seed-specific expression to study the function of genes in seed dormancy.

FT plays a central role in the regulation of flowering time (Turck *et al.*, 2008). Apart from flowering time control, *FT-like* genes also participate in fruit set, vegetative growth,

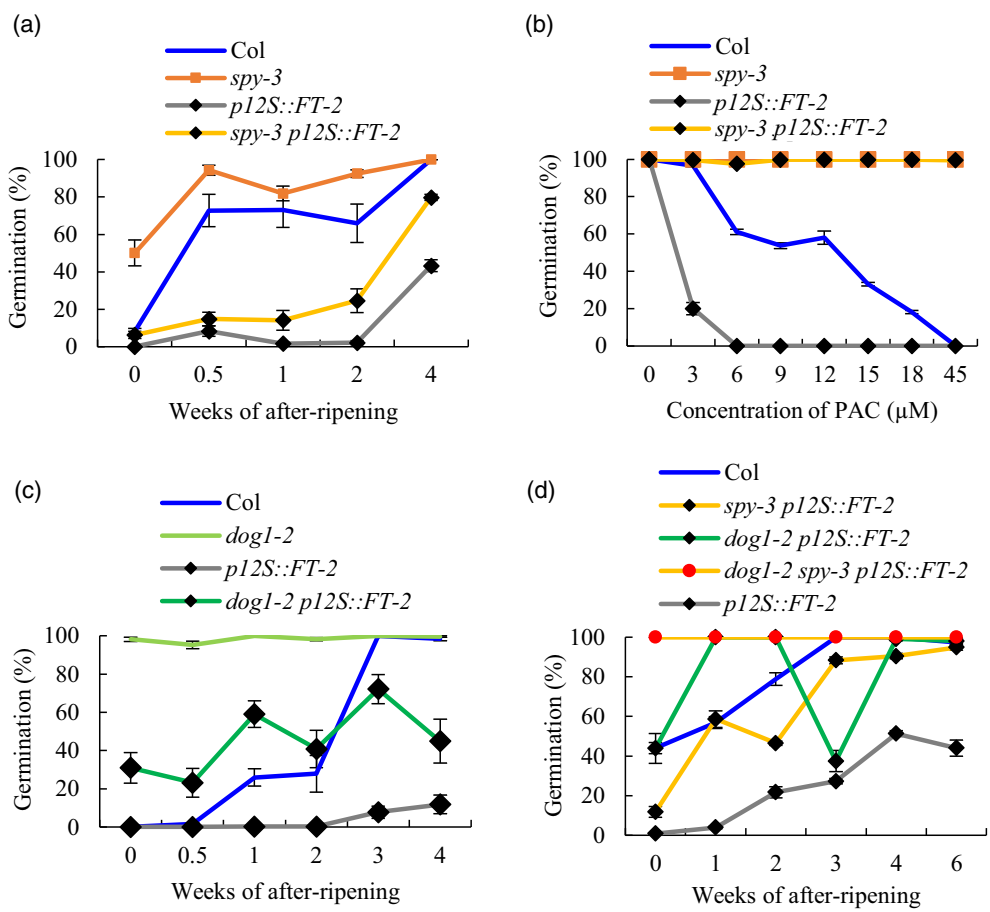

Figure 7. Genetic analysis of the relation between *SPY*, *DOG1*, and *FT*.
(a) Germination percentages of Col, *spy-3*, *p12S::FT-2*, and *spy-3 p12S::FT-2* during seed storage. (b) Sensitivity to PAC during seed germination of Col, *spy-3*, *p12S::FT-2*, and *spy-3 p12S::FT-2*. (c) Germination percentages of Col, *dog1-2*, *p12S::FT-2*, and *dog1-2 p12S::FT-2* during seed storage. (d) Germination percentages of Col, *spy-3 p12S::FT-2*, *dog1-2 p12S::FT-2*, *dog1-2 spy-3 p12S::FT-2*, and *p12S::FT-2* during seed storage. Percentages are averages (±SE) based on five to eight individual plants for each line.

stomatal control, and tuberization (Pin and Nilsson, 2012). It has been reported that FT plays a negative role in seed dormancy regulation by the transduction of maternal temperature in a L*er* background (Chen *et al.*, 2014). In the present study, we report that ectopic expression of *FT* in seeds of the Col background causes increased seed dormancy in a dose-dependent manner (Figure 1a,b), implying that FT positively regulates seed dormancy. This difference could be caused by the genotypic background. Moreover, increased seed dormancy of the *ft* mutant could be indirectly caused by its regulation of FLC since FT could not be detected in the Col wild-type seeds (Figure 1d). However, increased seed dormancy of *FT* ectopic expression lines could result from the direct function of FT by its interaction with unknown factors in seeds. In order to further testify that ectopic expression of *FT* directly results in increased seed dormancy, independent of its flowering time effects, we created *pNtCAT1::FT* transgenic lines. The *NtCAT1* promoter mainly drives expression in endosperm tissue (Suzuki *et al.*, 1995). We showed that *pNtCAT1::FT* transgenic seeds had the same dormancy level as Col despite flowering as early as dormant *p12S::FT* lines (Figure S2f,g). We conclude that ectopic expression of *FT* in seed directly results in increased seed dormancy (Figure 1e). This result also suggests that the effect of FT on dormancy might be independent from its effect on flowering time, which is consistent with the results reported by Chen *et al.* (2014).

Tyr85 of FT has a pivotal role in the promotion of flowering. In a previous study, the substitution of Tyr85 to His, the corresponding 88th amino acid in TFL1, resulted in function conversion of FT in flowering time regulation (Hanzawa *et al.*, 2005). In the present study, we report that *p12S::FT*(*Y85H*) transgenic lines showed enhanced seed dormancy without altered flowering time (Figure 3a–c; Figure S3a–c). This indicates that the Y85H mutation does not eliminate the function of FT in enhancing seed dormancy. Y85 is located at the potential ligand-binding pocket on the surface of the predicted three-dimensional structure of FT and this site was suggested to participate in protein interaction (Hanzawa *et al.*, 2005; Ho and Weigel, 2014). One hypothesis is that both FT and TFL1 may interact with the same partner (Abe *et al.*, 2005; Hanano and Goto, 2011; Resentini *et al.*, 2015; Taoka *et al.*, 2011). The key residues, namely Y85 in FT and H88 in TFL1, can determine the function of the complex, repression or activation (Hanzawa *et al.*, 2005). However, this hypothesis has not been confirmed at the molecular level yet. In other studies, it has been observed that both FT and TFL1 interact with the same members of 14-3-3 or TEOSINTE BRANCHED1, CYCLOIDEA, PCF (TCP) families with similar or different binding affinities (Ho and Weigel, 2014). However, the biological and molecular functions of most of these interactions are still unclear. Overall, it is still not completely understood at the molecular level how FT and TFL1 function as opposite factors in flowering time control. The ectopic expression of *FT*, *FT*(*Y85H*), and *TFL1* can all result in enhanced seed dormancy. They may all regulate seed dormancy by interacting with the same protein (complex) with different binding affinities. This would be consistent with our observation that higher expression levels are required for *FT*(*Y85H*) and *TFL1* compared to *FT* to induce similar dormancy enhancement. Among the binding partners of FT and TFL1 mentioned above (Ho and Weigel, 2014), TCP14 and TCP15 showed higher binding affinity with FT than TFL1 and have been suggested to participate in seed dormancy and germination regulation through an unknown molecular mechanism (Resentini *et al.*, 2015; Tatematsu *et al.*, 2008). We hypothesize that interaction with these TCP proteins might explain the similar role of FT and TFL1 in seed dormancy regulation.

Enhanced sensitivity of seed germination to PAC suggests that the GA pathway could be involved in the regulation of seed dormancy mediated by FT (Figure 5a–e). Our transcriptional analysis showed that several important genes involved in GA biosynthesis have significantly reduced transcript levels in seeds overexpressing *FT* (Figure 6a). Additionally, some GA-related genes, *EXPs* and *XTHs*, also showed significantly decreased transcript levels (Figure S5b). Moreover, bioactive GA_1, GA_3, GA_4, and GA_7 all showed declined contents in *p12S::FT-6* seeds imbibed for 24 h (Figure 6c). These results suggest that FT may regulate GA biosynthesis to modify seed germination. GA may enhance the growth of the radicle to break through the seed coat to improve seed germination, because stripping of the seed coat partially recovered the dormancy phenotype of the GA synthesis mutant *ga1-3* (Debeaujon and Koornneef, 2000; Silverstone *et al.*, 1997; Telfer *et al.*, 1997). The breaking of the root through the seed coat during germination is suggested to be associated with factors involved in cell wall loosening, for example, EXPs, XTHs, *etc.* (Bewley, 1997; Koornneef *et al.*, 2002). Moreover, previous studies in tomato (*Solanum lycopersicum*) seeds have shown that the presence of GA could upregulate the expression of multiple genes such as *EXPs*, *XTHs etc.*, and affect radicle release during germination (Chen and Bradford, 2000; Chen *et al.*, 2001; Nonogaki *et al.*, 2000). In the present study, we found that 100% of the dissected embryos of *FT* transgenic seeds with deep dormancy could germinate by peeling off seed coats (Figure 2b). Therefore, we propose that the ectopic expression of *FT* can weaken the GA signal by affecting the synthesis of GA and the expression of downstream genes in the GA signal pathway. Subsequently, the capability of the radicle to break through the seed coat is decreased, leading to a deeper dormancy of *FT* transgenic seeds. Genetic analysis of seed germination sensitivity of *spy-3 p12S::FT-2* to PAC further

confirms that the GA pathway is involved in *FT*-regulated seed germination, since SPY is epistatic to FT in seed germination in response to PAC (Figure 7b).

DOG1 is one of the most important specific seed dormancy regulators identified so far (Bentsink *et al.*, 2006; Nakabayashi *et al.*, 2012; Née *et al.*, 2017). The DOG1 protein is highly expressed in seeds and its level directly determines the depth of dormancy and germination time (Nakabayashi *et al.*, 2012). It is interesting that the expression level of *DOG1* was significantly increased in *p12S::FT* transgenic lines (Figure 6b), indicating that DOG1 might also be involved in FT-mediated seed dormancy. DOG1 can promote GA catabolism during maturation by GA2ox6 since *GA2ox6* showed decreased expression in the *dog1-2* mutant (Kendall *et al.*, 2011). DOG1 can also inhibit the expression of GA-regulated genes encoding cell wall-remodeling proteins in a temperature-dependent manner (Graeber *et al.*, 2014). These findings imply that there is a crosstalk of DOG1 with the GA pathway to regulate seed dormancy and germination. However, the GA pathway has relatively independent mechanisms to regulate seed dormancy and germination since the *dog1 ga1-3* double mutant seeds still require GA for germination although less so than the wild type (Bentsink *et al.*, 2006). Recently, a more direct mechanism by which DOG1 influences dormancy and germination was identified. The DOG1 protein can bind to and inhibit the action of two PP2C phosphatases, ABA HYPERSENSITIVE GERMINATION 1 (AHG1) and AHG3, to induce seed dormancy (Née *et al.*, 2017; Nishimura *et al.*, 2018). The mechanisms by which the DOG1 and GA pathways regulate seed dormancy and germination are either independent or overlapping. Therefore, impairment of either pathway might not completely recover the dormancy phenotype of *FT* transgenic lines. Indeed, this is consistent with what was observed in the genetic analysis of *spy-3 p12S::FT-2* and *dog1-2 p12S::FT-2* combinations (Figure 7a,c). However, genetic analysis of the triple mutant *dog1-2 spy-3 p12S::FT-2* showed that knockout of both SPY and DOG1 could completely recover the seed dormancy phenotype of *p12S::FT-2*. This implies that the dormancy phenotype of *FT* transgenic lines requires the presence of at least one of both DOG1 and GA pathways (Figure 7d).

Overall, significantly enhanced seed dormancy could be achieved when FT was highly expressed in seeds driven by the 12S promoter. The molecular mechanism involves the GA and DOG1 pathways. DOG1 mainly functions in induction and maintenance of dormancy (Bentsink *et al.*, 2006; Nakabayashi *et al.*, 2012; Nonogaki, 2019; Soppe and Bentsink, 2020), while GA accelerates germination initiation (Davière and Achard, 2013). Therefore, *FT* may regulate both dormancy and germination. Our study shows that ectopic expression of *FT* in seeds influences both flowering time and germination behavior. Interestingly, these effects can be separated by modifications of the FT protein, which could be a promising tool for molecular crop design.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

Arabidopsis thaliana (L.) Heynh. ecotype Columbia (Col) was used. The mutant *rgl2-13* was ordered from the Nottingham Arabidopsis Stock Centre (NASC, Nottingham, UK) (http://arabidopsis.info/). Primers for genotyping were designed using the SIGNAL T-DNA verification primer design program. The *spy-3* mutant is mutated at the 593rd amino acid with Gly converted to Ser (Qin *et al.*, 2011). The *dog1-2* mutant has two transversions of cytosines to adenines at the 332th and 334th sites of the coding sequence (CDS), leading to a premature termination of the DOG1 protein. For planting, seeds were imbibed at 4°C for 3–4 days and then sown in soil. Plants were grown in the greenhouse under a 16/8 h light/dark photoperiod at 18–22°C.

Germination assay

Germination tests were performed according to Alonso-Blanco *et al.* (2003). All germination experiments were performed on filter paper in 6-cm Petri dishes. Each experiment was repeated at least three times. In each independent experiment, each genotype had five to eight replicates from different individual plants, and 50–100 seeds were used for each replicate. The average germination percentage was determined after 7 days of incubation in a chamber (25°C, 16 h light at an intensity of 80–90 μmol m^{-2} sec^{-1}). Filter papers were soaked with 600 μL water. Germination percentages were calculated after 7 days.

Hormone sensitivity test

PAC was used as GA biosynthesis inhibitor. Filter papers were soaked with either water or solutions of PAC or ABA. Seeds were stored for about 3 months after harvest to break dormancy as much as possible, imbibed in soaked filter papers at 4°C for 3–4 days, and then transferred to normal germination conditions (25°C, 16 h light at an intensity of 80–90 μmol m^{-2} sec^{-1}). The germination percentages were calculated after 7 days.

Constructs and plant transformation

The pLEELA vector is a derivative of pJawohl3-RNAi (GenBank accession number AF404854). We used the pLEELA vector as the backbone and removed the double 35S promoters from pLEELA with *Asc*I and *Stu*I endonucleases. The primers 12S-F (GGCGCGCCAACTTAAGAGCTTATGATG) and 12S-R (AGGACCTTTCTTTTTGTTTGTTGTGAG) were used to clone the promoter of the *At4g28520* gene (1.85 kb upstream from ATG). The underlined nucleotides indicate the restriction sites of *Asc*I and *Stu*I, respectively. The 1.85-kb product was cloned into the pEASY vector and then digested by *Asc*I and *Stu*I. Then the gel extraction-purified fragment was ligated into the pLEELA fragment by T4 ligase to construct 12S-pLEELA. Successful construction of the 12S-pLEELA vector was confirmed by sequencing. We used the gene-specific primers FT-F (CACCTGTTCAAGATCAAAGATGTC) and FT-R (GCATCATCACCGTTCGTTACTC) to clone the *FT* CDS. The PCR product was cloned into the pENTR-D TOPO cloning vector by TOPO reaction and then cloned into 12S-pLEELA by LR reaction. The pNtCAT1::FT subclone was constructed with pDONR207-NtCAT1 and PAN3-FT by LR reaction. *NtCAT1* was cloned from the 2.7-kb fragment upstream of the *catalase* gene in castor bean

(*Ricinus communis*) (Suzuki *et al.*, 1995). It was shown to be specifically expressed in endosperm during seed germination in transgenic tobacco (*Nicotiana benthamiana*) seeds, in the upper parts of hypocotyl and cotyledons after germination, but not in leaves (Nakabayashi *et al.*, 2012; Suzuki *et al.*, 1995). The promoter was subcloned into pDONR207 by Nakabayashi (Nakabayashi *et al.*, 2012). The PAN3 vector was a derivative of pGreenII 0029 (GenBank accession number EU048864) (Nakabayashi *et al.*, 2012). The specific primers used to clone the *FT* CDS to PAN3 were as follows: FT-*Hin*dIII-F: AAGCTTATGTCTATAAATATAAGAGACC and FT-*Xho* I-R: CTCGAGCTAAAGT-CTTCTTCCTCCG. The underlined nucleotides indicate the restriction sites of *Hin*dIII and *Xho*I, respectively. The recombinant plasmids were introduced into Col wild type by infiltration with *Agrobacterium tumefaciens* strain GV3101 pm90RK (Clough and Bent, 1998). Transformed Arabidopsis lines were selected on the basis of their ability to survive after spraying twice with 150 mg L^{-1} BASTA. The 3:1 segregating transformants were selected on MS medium containing 10 mg L^{-1} DL-phosphinothricin. T3 homozygous transgenic plants were used for phenotypic analysis. *FT* transcript levels in freshly harvested dry seeds of transgenic plants were checked by Q-PCR.

Q-PCR analysis

Samples were taken from seeds isolated from 10-daf and 15-daf siliques and from freshly harvested seeds imbibed for 0, 6, 12, or 24 h in water in a chamber (25°C, 16 h light at an intensity of 80–90 μmol m^{-2} sec^{-1}). Total RNA was isolated using the Plant Total RNA Purification Kit (GeneMark, Taizhong, China, or TIANGEN, Beijing, China). cDNA was synthesized using the Fast King RT Kit (with gDNase) (TIANGEN). Subsequently, the cDNA was diluted 5–10 times. KAPA SYBR® FAST qPCR Kit Master Mix was used for Q-PCR. *ACTIN8* served as the internal standard. Seed pools from at least three individual plants were sampled as one biological replicate. Three biological replicates were included for each genotype. The statistical analysis was performed using the unpaired, two-tailed Student *t*-test to estimate the differences between *p12S::FT* transgenic lines and wild type. The presented values are averages (± standard error [SE]) from three biological replicates. It was hard to obtain a reliable Ct value of *FT*/*FT(Y85H)* and *TFL1* in Col seeds due to their low native expression, so we set this value to zero when we made charts. Q-PCR primers are listed in Table S1.

TTC assay

TTC staining of Arabidopsis seeds was performed as previously described (Debeaujon *et al.*, 2000). Aqueous solution of TTC (1% w/v) (Bioroyee, Beijing, China) was used, and seeds were placed at 30°C in darkness for 2–7 days.

Site-directed mutagenesis

The primers used to mutate FT Tyr85 were previously reported by Hanzawa *et al.* (2005). The Fast Mutagenesis System (TransGEN, Beijing, China) was used. Five nanograms of pENTR-FT (CDS) plasmid was used as template, forward and reverse primers were added to a final amount of 0.2 μM, 25 μL 2× TransStart FastPfu PCR Supermix was added, and distilled water was added to the reaction system to reach a total volume of 50 μL. The PCR product was digested with DMT enzyme to remove the non-mutated template and then transformed into DMT competent cells.

GUS staining

Embryos and testa (containing endosperms) were dissected from seeds at different developmental stages under a stereo microscope. Siliques were marked using thin threads when the flower just bloomed, as the first daf. Samples from *p12S::GUS* or *p35S::GUS* transgenic lines were immersed in GUS staining solution (Willemsen *et al.*, 1998) and vacuumed for 20 min, followed by incubation at 37°C for 3 h. After staining, samples were washed three times with distilled water and transferred into 70% ethanol. Images were taken by a Nikon 80i digital camera.

Measurement of bioactive GAs

Fresh dry seeds were imbibed in the growth chamber (16/8 h light/dark, 21 ± 1°C) for 24 h before sampling. Samples of 500 mg imbibed seeds were used. Quantification of GAs was performed using nano-liquid chromatography–electrospray ionization–quadrupole time-of-flight–mass spectrometry (nano-LC-ESI-Q-TOF-MS) as described previously (Chen *et al.*, 2012; Jiang *et al.*, 2016).

Protein extraction and immunoblotting

Twenty milligrams of dry seeds were collected and proteins were extracted by previously described methods (Wang *et al.*, 2016). Protein was extracted with a buffer containing 6 M urea, 2 M thiourea, 0.2% (v/v) Triton X-100, 0.2% (w/v) sarcosyl, and 2 mM dithiothreitol in 100 mM Tris-Cl, pH 7.5. The mixture was shaken for 30 min at 4°C and the supernatant was collected after centrifugation at 4°C. This procedure was repeated twice. The final supernatant was retained as total protein samples of seeds. The protein concentration was measured by Bradford dye reagent (Bio-Rad, Hercules, CA, USA) and BSA was used as a standard. Proteins were separated by 10% SDS-PAGE (about 20–30 mg protein per well) and transferred to polyvinylidene fluoride membranes (Merck Millipore, Billerica, MA, USA) by traditional semi-dry transfer using the TE77XP blot module (Hoefer, San Francisco, CA, USA). Proteins were detected using anti-FT (1:1000 dilution; al-20, Santa Cruz Biotech, Dallas, TX, USA) and a horseradish peroxidase-conjugated secondary antibody. Protein bands were visualized with Super ECL plus substrate (Applygen, Beijing, China) and imaged with a chemiluminescence imaging analysis system (Tanon-5200, Gelcap software).

Absolute quantitative PCR

Firstly, a standard curve was drawn as follows. Standard samples were produced using linearized plasmid fragments containing *FT* CDS or *TFL1* CDS fragments or fresh PCR product containing the *ACTIN8* CDS. Concentrations of these standard samples were determined by Nanodrop. Standard samples were diluted 10-fold six to eight times. Ct values were presented on the *x*-axis and $\log_{10}$(copy number) values were presented on the *y*-axis. Linear equations were fitted in Excel. For *FT*: $y = -3.6346x + 35.986$, $R^2 = 0.9985$; for *TFL1*, $y = -3.2207x + 33.517$, $R^2 = 0.993$; and for *ACTIN8*, $y = -2.9443x + 32.072$, $R^2 = 0.9819$. Secondly, the Ct values for *FT*, *TFL1*, and *ACTIN8* obtained from cDNA samples of *p12S::FT-2* and *p12S::TFL1-8* were inserted into the formulas to calculate the absolute number of copies of each gene in respective transgenic lines. Finally, the absolute amounts of *FT* and *TFL1* were normalized to *ACTIN8*. Significant differences were determined using Student's *t*-test (*$P < 0.05$, **$P < 0.01$).

ACCESSION NUMBERS

Gene IDs relevant to this paper are as follows: *CPS* (*At4g02780*), *KS* (*At1g79460*), *KO* (*At5g25900*), *KAO1* (*At1g05160*), *FT* (*At1g65480*), *TFL1* (*At5g03840*), *RGL2* (*At3g03450*), *SPY* (*At3g11540*), *DOG1* (*At5g45830*), *GA3ox1*

(*At1g15550*), *GA3ox2* (*At1g80340*), *GA20ox1* (*At4g25420*), *GA20ox2* (*At5g51810*), *GA2ox2* (*At1g30040*), *GA2ox6* (*At1g02400*), *EXP1* (*At1g69530*), *EXP2* (*At5g05290*), *EXP3* (*At2g37640*), *EXP8* (*At2g40610*), *EXP9* (*At5g02260*), *EXP10* (*At1g26770*), *EXP15* (*At2g03090*), *EXP20* (*At4g38210*), *XTH9* (*At4g03210*), *XTH15* (*At4g14130*), *XTH19* (*At4g30290*), and *ACTIN8* (*At1g49240*).

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AUTHOR CONTRIBUTIONS

FC and YL designed the research. FC performed the experiments. FC and YL analyzed the data. XL and JX contributed to RNA extraction and Q-PCR experiments. WL, ZW, HC, YL, and YL contributed reagents/materials/analysis tools. FC, ZW, HC, YL, WJJS, and YL participated in discussions and contributed to the writing of the article. FC, WJJS, and YL wrote the article.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All relevant data can be found within the manuscript and its supporting materials.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Expression pattern of the 12S promoter.

Figure S2. Germination and flowering phenotypes of different FT transgenic lines.

Figure S3. Flowering time analysis of *p12S::FT(Y85H)* and *p12S::TFL1* transgenic lines.

Figure S4. Sensitivity of seed germination to ABA.

Figure S5. Gene expression analysis by Q-PCR.

Figure S6. Genetic analysis of the relation between *RGL2* and *FT*.

Table S1. List of Q-PCR primers.

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