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Effect of *FLOWERING LOCUS C* on seed germination depends on dormancy

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Abstract. *FLOWERING LOCUS C* (*FLC*) has a major regulatory role in the timing of flowering in *Arabidopsis thaliana* (L.) Heynh. and has more recently been shown to influence germination. Here, we investigated the conditions under which *FLC* influences germination, and demonstrated that its effect depends on the level of primary and secondary dormancy and the temperature of seed imbibition. We tested the germination response of genotypes with different degrees of *FLC* activity over the course of after-ripening and after secondary dormancy induction by hot stratification. Genotypes with high *FLC*-activity showed higher germination; this response was greatest when seeds exhibited primary dormancy or were induced into secondary dormancy by hot stratification. In this study, which used less dormant seeds, the effect of *FLC* was more evident at 22°C, the less permissive germination temperature, than at 10°C, in contrast to prior published results that used more dormant seeds. Thus, because effects of *FLC* variation depend on dormancy, and because the range of temperature that permits germination also depends on dormancy, the temperature at which *FLC* affects germination can also vary with dormancy. Finally, we document that the effect of *FLC* can depend on *FRIGIDA* and that *FRIGIDA* itself appears to influence germination. Thus, pleiotropy between germination and flowering pathways in *A. thaliana* extends beyond *FLC* and involves other genes in the *FLC* genetic pathway.

Additional keywords: after-ripening, conditional dormancy, dormancy, germination, *FRIGIDA*, pleiotropy, temperature.

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Introduction

Plant life cycles comprise multiple developmental transitions. In annual plants two critical transitions are from seed to seedling, or germination, and from vegetative to reproductive status, or flowering (Chouard 1960; Kalisz and Wardle 1994; Nordborg and Bergelson 1999; Burghardt *et al*. 2015; Springthorpe and Penfield 2015). The seasonal timing of these transitions, or phenology, strongly influences the performance of plants in novel environments (Walther *et al*. 2002; Menzel *et al*. 2006; Parmesan 2006; Chuine 2010; Willis *et al*. 2014). Predicting how organisms respond to environmental change requires both understanding the genes that regulate these major phenological transitions and the environments under which these genes function.

Because germination precedes all other developmental transitions in plants, its timing has been demonstrated to be under strong natural selection (Kalisz 1986; Donohue *et al*. 2005, 2010; Huang *et al*. 2010; Akiyama and Ågren 2014). In *Arabidopsis thaliana* (L.) Heynh., germination and dormancy, and the genes that control them, can be subject to variable natural selection and contribute to local adaptation (Kronholm *et al*. 2012; Montesinos-Navarro *et al*. 2012). The seasonal timing of seed germination is strongly determined by dormancy; as seeds lose dormancy, they are able germinate over a wider range of temperatures ('conditional dormancy'), leading to temperature-dependent germination that depends on dormancy status (Bewley 1997; Baskin and Baskin 2014). It is the change in temperature-dependent germination over time (as dormancy is lost) that determines the seasonal timing of germination. Primary dormancy is induced during seed maturation, and it prevents the germination of maturing and freshly dispersed seeds. Primary dormancy is under strong maternal control and can be altered according to the environmental conditions during seed maturation (Gutterman 2000; Kendall and Penfield 2012; Burghardt *et al*. 2015; MacGregor *et al*. 2015). Primary dormancy is gradually lost over time through a process known as after-ripening. However, if favourable conditions for germination are not available even after a period of after-ripening, non-dormant seeds can cycle back into secondary dormancy, and secondary dormancy can be enhanced by unfavourable conditions (Auge *et al*. 2015). The hormone abscisic acid induces and maintains dormancy, and gibberellins promote germination (Bewley 1997; Finch-Savage and Leubner-Metzger 2006; Holdsworth *et al*. 2008*a*, 2008*b*; Footitt *et al*.

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2011). The biosynthesis, catabolism, and signalling of these hormones is regulated by several genes implicated in dormancy induction and maintenance (Holdsworth *et al.* 2008*b*; Rodríguez *et al.* 2009; Footitt *et al.* 2011; Kendall *et al.* 2011; Mendiondo *et al.* 2010; Nakamura *et al.* 2011; Graeber *et al.* 2012; Nakabayashi *et al.* 2012; Vaistij *et al.* 2013). Although primary and secondary dormancy both depend on these phytohormones, it is unclear the degree to which the same signalling or integrator genes regulate both primary and secondary dormancy.

Flowering time strongly influences fitness in many plants, because it determines whether and how long plants can produce seeds before the end of the growing season as well as the conditions under which they do so (see reviews by Munguía-Rosas *et al.* 2011; Ehrlén 2015). Much is known about the genetic regulation of flowering and its response to seasonal environmental cues (Michaels and Amasino 1999, 2001; Simpson and Dean 2002; Gazzani *et al.* 2003; Michaels *et al.* 2003, 2005; Ausin *et al.* 2005; Werner *et al.* 2005; Bäurle and Dean 2006; Dennis and Peacock 2007). In particular, multiple genetic pathways of flowering regulation converge onto the central flowering regulator, *FLOWERING LOCUS C* (*FLC*). The vernalisation and autonomous pathways directly regulate the expression of *FLC*, which suppresses the floral integrators that promote flowering, *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF CONSTANS OVEREXPRESSION 1* (*SOC1*) (Lee *et al.* 2000; reviewed by Mouradov *et al.* 2002). The photoperiod and gibberellin pathways are integrated directly downstream of *FLC* to regulate those same floral integrators. Thus *FLC* and genes regulated by it are central regulators of flowering time.

One pertinent question concerns the degree to which multiple life-stage transitions are genetically independent, or alternatively, the degree to which they share major genetic pathways of regulation. On the one hand, pleiotropy can result in adaptively integrated phenotypes, such that a single allelic change alters multiple phenotypes in an adaptively coordinated manner (Atchley 1984; Wagner 1995; Murren 2002; Pigliucci 2003; Brakefield 2006; Wagner *et al.* 2008). In contrast, pleiotropy may impose constraints on the expression of optimal phenotypes, if a favourable change in one phenotype is accompanied by unfavourable changes in others (Fisher 1958; Atchley 1984; Wagner 1988; Barton 1990; Crespi 2000; Orr 2000; Griswold and Whitlock 2003). Here we investigate pleiotropy between germination and flowering pathways in *A. thaliana*, focusing on the major flowering gene, *FLOWERING LOCUS C* (*FLC*).

FLC was first identified as master regulator of flowering time, and has since been shown to be a functionally conserved throughout flowering plants (Michaels and Amasino 1999; Ream *et al.* 2012). In flowering, the expression is regulated by several genes, including *FRIGIDA* (*FRI*) and genes in the vernalisation and autonomous pathways. *FLC*'*s* repression of the promoters of flowering, *FT* and *SOC1*, is relieved by an epigenetic switch in the chromatin structure around *FLC* (Dennis and Peacock 2007; Choi *et al.* 2009). This switch is induced through a prolonged period of winter cold (known as vernalisation), and plants with high expression of *FLC* during the pre-vernalisation stage require cold to induce flowering, resulting in flowering during the spring, only after winter chilling. Extensive natural variation in *FLC* exists, including geographic clines, and such geographic variation has been interpreted to reflect a history of natural selection on flowering time (Sheldon *et al.* 2000; Gazzani *et al.* 2003; Michaels *et al.* 2003; Caicedo *et al.* 2004; Stinchcombe *et al.* 2004, 2005; Le Corre 2005; Shindo *et al.* 2005, 2006; Korves *et al.* 2007; Coustham *et al.* 2012; Sánchez-Bermejo *et al.* 2012). Geographic patterns of variation in *FLC* are contingent on natural variation in *FRI* (Caicedo *et al.* 2004), since *FRI* is necessary for *FLC* expression at the pre-reproductive stage in several ecotypes (Clarke and Dean 1994; Koornneef *et al.* 1994; Lee and Amasino 1995; Johanson *et al.* 2000; Gazzani *et al.* 2003). Multiple independent losses of *FRI* function have also been discovered, leading to a lack of vernalisation requirements for flowering (Le Corre *et al.* 2002; Shindo *et al.* 2005). This variation in *FRI* has also been interpreted as being maintained by geographically variable natural selection on flowering (Le Corre *et al.* 2002; Shindo *et al.* 2006; Strange *et al.* 2011), although other pleiotropic effects of *FRI* have been discovered, including inflorescence architecture and resistance to drought (Scarcelli *et al.* 2007; Lovell *et al.* 2013).

FLC has more recently been shown to be associated with germination in *A. thaliana* (Chiang *et al.* 2009). Seeds carrying an introgressed high *FLC*-allele or an overexpressing gene construct had a significantly higher proportion of seed germination (Chiang *et al.* 2009). This effect depended on the temperature at which seeds were imbibed; however, with the contribution of *FLC* being manifest much more strongly when seeds were imbibed at 10°C than at 22°C. Reciprocal crosses showed that the maternal *FLC* allele had larger effects on seed germination than the paternal allele, corresponding with a peak of *FLC* expression during late stages of seed development (Chiang *et al.* 2009) when primary dormancy is induced (Bewley 1997; Kucera *et al.* 2005). High *FLC* expression during silique development, moreover, was associated with reduced expression at the silique stage of downstream targets of the *FLC*-mediated flowering pathway – *FT*, *SOC1* and *AP1*. Seeds from those siliques, in turn, exhibited early upregulation of the abscisic acid degradation gene *CYP707A2* and the later increase of the gibberellic acid synthesis gene *GA20ox1*. Thus, high *FLC* expression in maturing siliques appears to enhance germination, possibly via some of the same downstream genes that are involved in flowering regulation. The degree to which upstream regulators of *FLC* are also involved has not been tested.

Studies using seeds buried under natural conditions also identified *FLC* as a gene that cycles seasonally with dormancy, along with other genes known to be involved in dormancy regulation (Footitt *et al.* 2011, 2014). Therefore, *FLC* may be involved not only in primary dormancy but also in secondary dormancy cycling, although this has not yet been tested directly.

Here, we tested the effect of *FLC* on germination using different natural *FLC* alleles, mutants, and an *FLC*-RNAi knockdown of *FLC* activity. We evaluated the contribution of *FLC* to germination after different treatments that manipulated primary and secondary dormancy status. Specifically, we examined germination after different durations of dry after-ripening, which alleviates primary dormancy, and after different durations of a secondary-dormancy inducing treatment. We asked

the following questions. (1) Does germination of seeds with primary dormancy vary with direct genetic alteration of *FLC* activity? (2) Does *FLC* influence secondary dormancy induction? (3) To what extent does the effect of *FLC* on germination depend on the depth of primary and secondary dormancy and on temperature of seed imbibition? (4) And does the effect of *FLC* depend on the positive regulator of *FLC* expression, *FRI*?

Materials and methods

Plant material

To quantify the effect of *FLC* on germination of seeds in different dormancy states, we used high and low-expressing *FLC* alleles in two standard background ecotypes of *Arabidopsis thaliana* (L.) Heynh.: Landsberg *erecta* (L*er*) and Columbia (Col-0). In the L*er* background, we compared germination of the *FLC*-nonfunctional wild type to a high *FLC* allele in a near isogenic line (NIL), L*er*-FLC_{CVi} (Alonso-Blanco *et al*. 1998). Unlike other natural ecotypes, this *FLC* allele from the Cape Verde Island (CVi) ecotype exhibits *FLC* expression even without a functional *FRI* allele (Gazzani *et al*. 2003). We also generated an RNAi-knockdown of *FLC* expression of this high-*FLC* NIL (L*er*-FLC_{CVi} -RNAi; RNAi #2, in Supplementary Text 1 available as Supplementary Material to this paper). Also, see Supplementary Text 1 for a description of its construction and validation.

In the Col background, the wild-type *FLC* allele is functional, but it exhibits low expression in the absence of a strong *FRIGIDA* (*FRI*) allele. As such, we used lines with manipulations of both *FLC* and *FRI* to compare the germination behaviour between all possible combinations of *FLC* and *FRI* activity. For the *FLC* allele, we compared the functional wild-type FLC_{Col} allele to the nonfunctional *flc-3* mutant; for *FRI*, we compared the non-functional FRI_{Col} allele to a NIL with the introgressed, high *FRI*-expressing allele from the San Feliu ecotype (Sf-2). A summary of all of the genotypes, with respective *FLC* and *FRI* alleles, is listed in Table 1. Seeds were kindly gifted by Scott Michaels and Maarten Koornneef, or purchased from The *Arabidopsis* Seed Stock Center at The Ohio State University.

Plant growth conditions

Unfortunately, we did not have the L*er*-FLC_{CVi}-RNAi knockdown available for Experiment 2. To provide seed donors, seeds were sown in 0.6% w/v agar plates, stratified at 4°C for 7 days in darkness, and then allowed to germinate at 22°C in a 12 h light cycle. Seedlings were then transferred to pots with potting soil (Metromix 360 soil, Scotts Sierra) and placed in EGC GCW-30 Plant Growth Chambers (Environmental Growth Chambers) under a 12 h light cycle at 22°C until bolting. The plants were then transferred to short days (8 h light/16 h darkness) at 15°C until seeds matured. Plants were fertilised every 14 days with a 300 g N L^{-1} nitrogen solution of Blossom Booster Fertiliser (JR Peters). Watering was withheld 1 week before harvest to induce the drying of siliques. Plants for Experiments 1 and 2 were grown in different batches but under the same conditions. For logistical reasons, plants for Experiment 2 were grown in two batches: the first batch provided fresh seeds that were used in germination assays 3 days after harvest. The second batch provided seeds that were after-ripened for 3 and 5 months. For each batch, harvest of all plants occurred on a common day. After harvesting, all seeds were dried at room temperature in a Clean Horizontal Secador Powered 115v Desiccator Cabinet (Bel-Art Products). Seeds for Experiment 1 and fresh seeds of Experiment 2 were used soon after harvest. After-ripened seeds for Experiment 2 were stored at 4°C until used. For the second experiment, because seeds with different after-ripening durations were harvested from different plants and stored under different conditions, the comparison across after-ripening durations is confounded with batch. However, the motivation for different after-ripening treatments was to manipulate the degree of primary dormancy; the batches did differ significantly in dormancy, as intended, even though we cannot attribute this difference solely to after-ripening.

Experimental conditions for germination assays

First (Experiment 1), we tested for effects of *FLC* variation in fresh seeds that exhibited some primary dormancy, and we further induced secondary dormancy in those seeds by imbibing them in the dark at 35°C ('hot stratification') for different durations. Fresh seeds (3 days after harvest) were sown in 35 mm Petri plates with 0.6% w/v agar-agar and either immediately hot stratified for 1, 3, 5 or 7 days in Percival Model GR41 LX incubation chamber (Percival Scientific Inc.) or were not hot-stratified. Plates were then incubated at 10°C or 22°C in a 12 h light/12 h dark cycle (PAR: 120–150 µmol m^{-2} s^{-1}) in EGC

Table 1. Genotypes used in the study

'L*er*' indicates the Landsberg *erecta* genotype. 'Col' indicates the Columbia genotype. 'L*er*-FLC_{CVi}' is the L*er* background with an *FLC* allele from the Cape Verde Island ecotype introgressed into it. 'Col-FRI_{Sf}' is the Col background with a *FRI* allele from the San Feliu-2 ecotype introgressed into it. '*flc-3*' is a knockout of the *FLC* gene (provided by Scott Michaels). See Supplementary Text 1 for more information on the RNAi construct

Genotype	FRI allele		FLC allele		ID	Expected FLC expression
L*er* wild type	L*er*	Weak	L*er*	Weak	L*er*	Low
L*er*-FLC_{CVi}	L*er*	Weak	CVi	Strong	L*er FLC*	High
L*er*-FLC_{CVi}-*RNAi*	L*er*	Weak	Construct	Knock down	L*er FLC-RNAi*	Low
Col wild type	Col	Weak	Col	Weak	*fri*/*FLC*	Low
Col *flc-3*	Col	Weak	Col	Knock down	*fri*/*flc*	Null
Col-FRI_{Sf}	Sf-2	Strong	Col	Weak	*FRI*/*FLC*	High
Col-FRI_{Sf} *flc-3*	Sf-2	Strong	Col	Knock down	*FRI*/*flc*	Null

Model GC8–2 Plant Growth Chambers (Environmental Growth Chambers). For all germination assays, 12 replicates (plates) were plated, for each treatment and genotype, with 20 seeds per plate. Twelve different maternal plants supplied seeds for the 12 replicate plates. Final germination proportion (seeds showing radicle protrusion/total number of viable seeds) was recorded at 14 days after transfer to light, when germination had reached a clear plateau.

Second (Experiment 2), to test the effect of *FLC* on secondary dormancy induction and whether it interacts with primary dormancy, we manipulated the duration of after-ripening and the duration of hot stratification, as above. Seeds were after-ripened for 3 days, 3 months, or 5 months. Seeds were plated and hot-stratified as above, and then incubated at 10, 22 or 31°C in a 12 h light/12 h dark cycle, as described above. Nine replicate plates (with each replicate from a different maternal plant) were plated for seeds after-ripened for 3 days and for 3 months; four replicate plates (maternal plants) were available for seeds after-ripened for 5 months. Final germination proportion was recorded at 14 days after transfer to light, when germination had reached a clear plateau.

Statistical analysis

We analysed the final proportion of seeds that germinated with logistic regression (PROC LOGISTIC in SAS 9.4; SAS Institute) using Fisher's scoring optimisation (ML) algorithm, and performed Type-III likelihood ratio tests. We used Firth's penalised likelihood to accommodate issues of quasi-separation caused by extreme germination proportions (0 or 100%) in some treatments. Quasi-separation is common in logistic/probit regression when certain combinations of predictor variables lead to all or nothing outcomes. Firth's bias-reduced penalised likelihood accommodates separation issues and was therefore employed in our regression model. We also used PROC GENMOD, but results were mainly concordant, so we present results only from PROC LOGISTIC.

For the first experiment using fresh seeds, we tested for effects of genotype ('Geno'), seed incubation temperature ('Temp'; 10 vs 22°C), and duration of hot stratification ('Hot strat'). All factors were fixed factors. We analysed the L*er* and Col backgrounds separately. For the L*er* background, L*er*-FLC_{CVi} was the reference genotype. In the Col background, a single reference genotype was not appropriate; we therefore compared functional versus non-functional *FLC* alleles on a functional *FRI* background (FRI/FLC vs FRI/*flc*) and on a non-functional *FRI* background separately (*fri*/FLC vs *fri*/*flc*). We likewise compared functional versus non-functional *FRI* alleles on both a functional (FRI/FLC vs *fri*/FLC) and non-functional *FLC* background (FRI/*flc* vs *fri*/*flc*) in supplementary analyses. Significance levels were Bonferroni corrected. To interpret significant interactions with incubation temperature, we next analysed each imbibition temperature separately. Because of significant Geno × Hot strat interactions, we subsequently tested for differences among genotypes in each combination of incubation temperature and duration of hot stratification.

For the second experiment, using seeds after-ripened for different durations, we tested for effects of Geno, seed incubation temperature ('Temp'; 10 vs 22°C), duration of hot stratification ('Hot strat'), duration of after-ripening ('AR') and all interactions, with all factors considered fixed factors. As before, we analysed the L*er* and Col genetic backgrounds separately, and made comparisons among genotypes as described above. A full model that included all interactions was analysed first to test for significant four-way interaction. To interpret interactions with duration of after-ripening, we next analysed each after-ripening treatment separately. To interpret significant interactions between genotype and imbibition temperature or duration of hot stratification at each after-ripening duration, we tested for differences between genotypes in each combination of treatments.

Results

Landsberg-erecta *background*

Low FLC *activity is associated with reduced germination in hot-stratified fresh seeds (Experiment 1)*

Seeds incubated at 10°C exhibited little dormancy compared with seeds incubated at 22°C, and differences among genotypes were therefore more (although not significantly more) pronounced at 22°C (Fig. 1*a*; and see Table S1, available as Supplementary Material to this paper). The low-*FLC* L*er* genotype had significantly lower germination than the high-*FLC* NIL (L*er*-FLC_{CVi}) at both imbibition temperatures (Tables 2, S2). However, at 10°C (in which all genotypes had high germination), the difference was more apparent after secondary dormancy induction by hot stratification (indicated by a significant Geno × Hot strat interaction; Table 2), although some of these differences were very small and not likely to be biologically significant. At 22°C in contrast, the L*er* genotype had lower germination than the L*er*-FLC_{CVi} genotype even without secondary dormancy induction by hot stratification, and the genotypes did not respond significantly differently to duration of hot stratification when they were subsequently imbibed at 22°C.

The L*er* genotype differs from the L*er*-FLC_{CVi} genotype in a region of chromosome that contains the *FLC* locus, but that region may also contain other genes. The RNAi knockdown of *FLC*; however, provides a direct test of whether disruption of *FLC* is causally associated with reduced germination. Although the RNAi knockdown of *FLC* (L*er*-FLC_{CVi} -RNAi) did not have germination as low as the L*er* background genotype (nor did it have *FLC* expression that was as low as the L*er* background; see Supplementary Text 1, Fig. S5), it had significantly lower germination than its control genotype (L*er*-FLC_{CVi}) when seeds were incubated at 22°C (Tables 2, S2; Fig. 1*a*). Therefore, silencing of *FLC* itself reduced germination, and germination differences between L*er* and L*er*-FLC_{CVi} are at least in part due to differences in *FLC* activity *per se*. Although these genotypes did not differ significantly in the degree to which they were induced into secondary dormancy (non-significant Geno × Hot strat interaction; Table 2), the RNAi-knockdown of *FLC* exhibited significantly lower germination than its control (L*er*-FLC_{CVi}) only when hot stratified (Fig. 1*a*). Therefore, reduced *FLC* activity significantly decreased germination, but this effect is manifest only when seeds exhibit some (secondary) dormancy

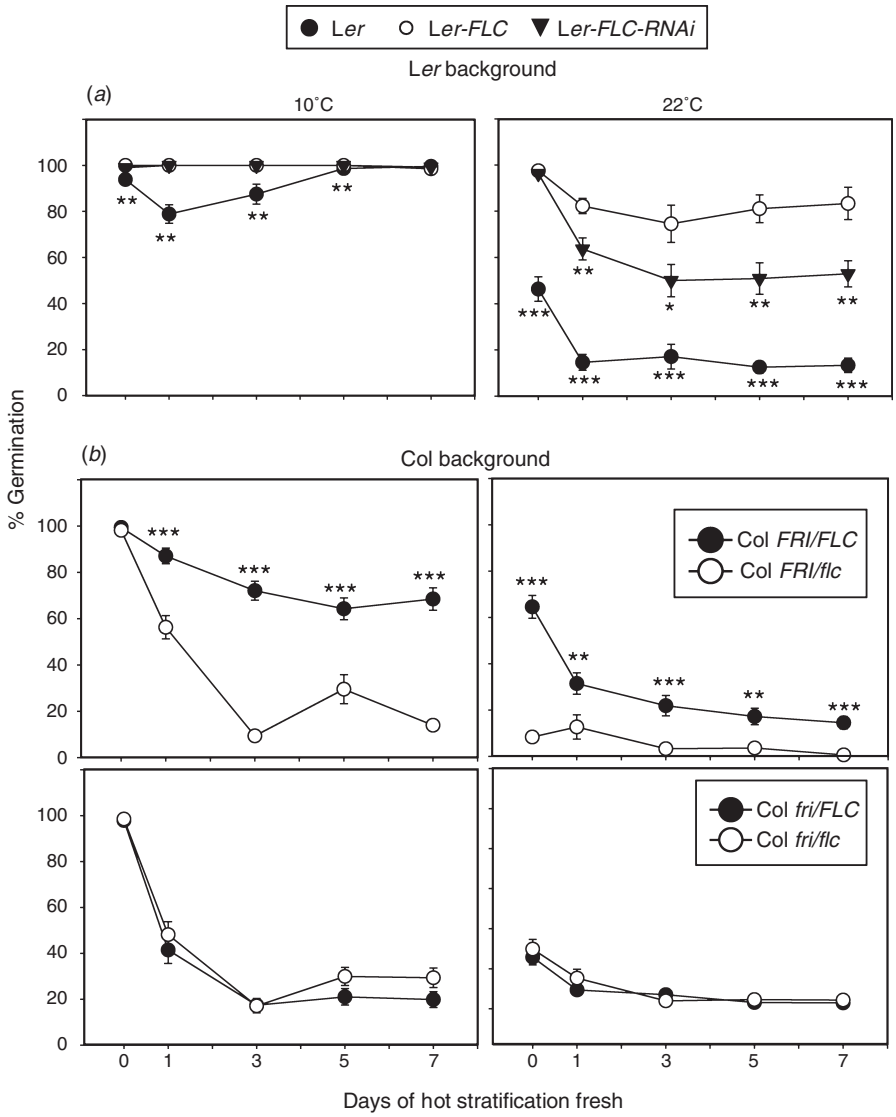

Fig. 1. Effect of *FLC* disruption in fresh seeds (Experiment 1) imbibed at 10°C (left) and 22°C (right). Mean germination percentages (±s.e.) are shown for fresh seeds given different durations of pre-incubation at 35°C ('hot stratification') to induce secondary dormancy. (*a*) Genotypes on the L*er* background; asterisks below the black circles indicate significant differences between the L*er* background and the high-*FLC* near isogenic line, and asterisks below the black triangles indicate significant differences between the L*er*-FLC_{CVi} NIL and the RNAi knock-down of that genotype. (*b*) Genotypes on the Col background. Significant differences between genotypes are based on Wald Chi-square values from logistic regression. Significant differences are indicated: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

and at temperatures that are not as conducive to germination (22°C).

The effect of FLC *on germination depends on primary dormancy (Experiment 2)*

The seeds used for Experiment 2 were slightly more dormant than those from Experiment 1, with fresh seeds exhibiting less germination. Seeds lost primary dormancy with longer periods of after-ripening (Fig. 2; Table S3). As before, in seeds that did not experience dormancy-inducing hot stratification (left-most points in Fig. 2), imbibition at 22°C was associated with less germination than imbibition at 10°C.

In seeds without the 35°C hot stratification, differences between the low-*FLC* L*er* genotype and the high-*FLC* L*er*-FLC_{CVi} genotype depended on the degree of primary dormancy and incubation temperature (significant Geno × AR × Temp interaction in Table S3 and significant Geno × Temp in some, but not all, after-ripening conditions in Table S4). Seeds imbibed at 10°C exhibited some dormancy when fresh, but not when after-ripened, and differences between the genotypes were of largest magnitude in fresh seeds (Fig. 2; Table S5). In seeds

Table 2. Effects of *FLC* disruption in fresh seeds imbibed at 10°C (upper) and 22°C (lower) in seeds from Experiment 1

Results of logistic regression to test for significant differences among genotypes ('Genos'), the number of days of hot stratification ('Hot strat'), and their interactions in each imbibition temperature separately. Analyses were conducted for each background separately (L*er* and Col), and specific genotype contrasts are indicated in the column headings. Results show the Wald Chi-square (χ^2) statistic from Type 3 results of logistic regression of the final proportion of seeds that germinated. Reference level was 0 days of hot stratification and specific genotype contrasts are indicated in the column headings. Significant differences are indicated: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. For individual genotype comparisons, boldface indicates significance after Bonferroni correction for multiple comparisons

		L*er*		Col	Col – effect of *FLC*		Col – effect of *FRI*	
	All genos	L*er*-FLC vs L*er*	L*er*-FLC vs RNAi	All genos	FRI/FLC vs FRI/*flc*	*fri*/FLC vs *fri*/*flc*	FRI/FLC vs *fri*/FLC	FRI/*flc* vs *fri*/*flc*
				10°C				
	χ^2 (df)	χ^2 (df)	χ^2 (df)	χ^2	χ^2	χ^2	χ^2	χ^2
Geno	52.36*** (2)	**24.62*** (1)	0.57 (1)	57.15*** (3)	**46.05*** (3)	1.90 (3)	**49.44*** (3)	1.72 (3)
Hot strat	4.03 (4)	2.88 (4)	**31.70*** (4)	241.74*** (4)	**110.75*** (4)	**145.58*** (4)	**84.45*** (4)	**177.07*** (4)
Geno × Hot strat	33.13*** (8)	**19.45*** (4)	7.71 (4)	26.62** (12)	**18.56**** (12)	1.67 (12)	2.80 (12)	10.56* (12)
				22°C				
Geno	224.44*** (2)	**192.72*** (1)	**20.7***	132.67*** (3)	**59.69*** (3)	0.77 (3)	**66.24*** (3)	**7.20**** (3)
Hot strat	61.12*** (4)	**27.72*** (4)	**40.24***	131.7*** (4)	**45.13*** (4)	**107.56*** (4)	**125.33*** (4)	**46.22*** (4)
Geno × Hot strat	9.03 (8)	2.35 (4)	2.15	21.08* (12)	**15.58**** (12)	3.64 (12)	0.79 (12)	9.6* (12)

imbibed at 22°C, the L*er* genotype gradually lost primary dormancy with increased after-ripening duration, while the L*er*-*FLC*$_{CVi}$ genotype had low dormancy even when fresh. As a consequence, differences among genotypes were most pronounced in fresh seeds, and those differences diminished as L*er* lost primary dormancy through after-ripening (Table S5). In seeds incubated in the light at 31°C, germination of both genotypes was extremely low in fresh seeds, and only the high-*FLC* L*er*-*FLC*$_{CVi}$ genotype was able to germinate even after 5 months of after-ripening (see Fig. S1, available as Supplementary Material to this paper). Because of the extremely low germination of seeds incubated at 31°C, therefore, differences among genotypes were only expressed in the least dormant seeds. In summary, differences in germination between high-*FLC* and low-*FLC* genotypes were only manifest at intermediate levels of primary dormancy; if dormancy was too low, both genotypes exhibited high germination, but if dormancy was too high and germination conditions too non-permissive, neither genotype was able to germinate.

High FLC *activity is associated with reduced secondary dormancy induction (Experiment 2)*

When seeds were imbibed at 10°C, the high-*FLC* L*er*-*FLC*$_{CVi}$ genotype was not induced into secondary dormancy, regardless of duration of after-ripening, whereas the low-*FLC* L*er* genotype was induced into secondary dormancy by hot stratification, especially when fresh (Fig. 2; Table S5). However, the response to secondary dormancy induction did not differ significantly between these genotypes when incubated at 10°C (non-significant Geno × Hot strat interaction in Tables S3 and S4). When seeds were imbibed at 22°C, hot stratification induced strong secondary dormancy. In fresh seeds at this temperature, both genotypes were induced into secondary dormancy by hot stratification, though the magnitude of this effect differed between seed batches and by hot stratification duration (Fig. 1*a* c.f. Fig. 2). In seeds after-ripened for 3 or 5 months, secondary dormancy was induced more strongly in the L*er* genotype than in the L*er*-*FLC*$_{CVi}$ genotype. Again, although the Geno × Hot strat interaction was not significant (Tables S3 and S4), differences among genotypes in after-ripened seeds were significant (and highly so) only in seeds that were induced into secondary dormancy by hot stratification (Table S5). Therefore, high *FLC* activity appears to be associated with less secondary dormancy induction by pre-incubation at hot temperature.

Columbia background

FLC *disruption causes decreased germination, but this difference is apparent only when* FRI *is active (Experiment 1)*

As in the L*er* background, germination proportions of fresh seeds in the Col background were greater when imbibed at 10°C than when imbibed at 22°C for all genotypes (Fig. 1*b*; Table S1). Hot stratification induced secondary dormancy, thereby reducing germination.

On an active *FRI* background (upper panels of Fig. 1*b*; Table S2), disruption of *FLC* reduced germination. When seeds were imbibed at 10°C, fresh seeds of both genotypes were able to germinate to high proportions, but seeds with disrupted *FLC* were induced into significantly stronger secondary dormancy by hot stratification (significant Geno × Hot-strat interaction in Table 2). At 22°C, seeds with disrupted *FLC* had lower germination even without secondary dormancy induction (Table S2). Therefore, as in the L*er* background, reduced *FLC* activity was associated with higher primary dormancy (expressed at 22°C) and a greater propensity to be induced into secondary dormancy (expressed at 10°C).

On an inactive *FRI* background (lower panels of Fig. 1*b*; Table 2, Tables S1 and S2), disruption of *FLC* had no effect on germination, and germination of those seeds resembled those of the *flc* mutant genotype when *FRI* was functional. Therefore, in the Col background, the effect of *FLC* on

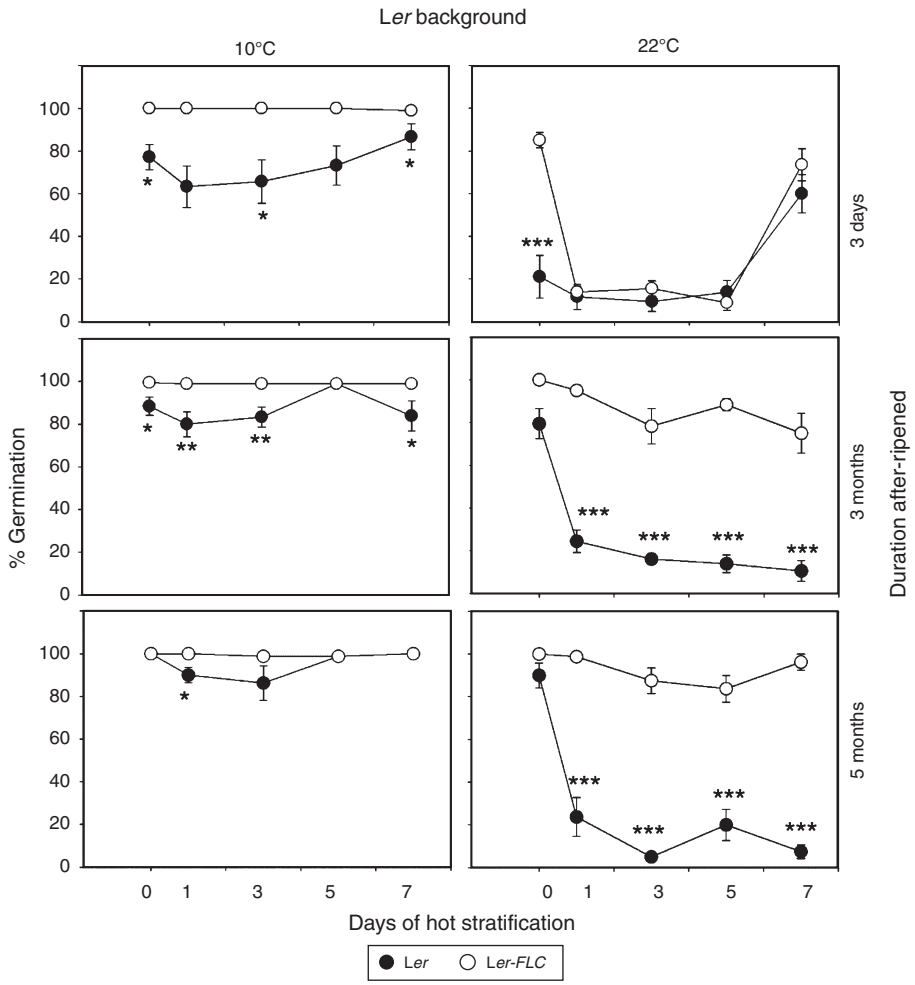

Fig. 2. Effect of *FLC* disruption over the course of after-ripening (Experiment 2) in the L*er* background. Mean germination percentages (±s.e.) are shown for seeds after-ripened for 3 days, 3 months and 5 months, given different durations of hot stratification to induce secondary dormancy (*x*-axis), and imbibed at 10°C (left) and 22°C (right). Significant differences between genotypes are based on Wald Chi-square values from logistic regression. Significant differences are indicated: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

germination was only manifest when *FRI* is active, suggesting that *FRI* is necessary for *FLC* activity, as is the case for flowering.

The effect of FLC *disruption on germination depends on primary dormancy (Experiment 2)*

As before, germination proportions were higher when seeds were incubated at 10°C than at 22°C, and seeds lost dormancy (germinated to higher percentages) as they after-ripened (Figs 3, 4, apparent at 22°C; Table S3).

On the functional *FRI* background, in seeds that did not experience hot stratification (left-most points in Fig. 3), the effect of *FLC* disruption was manifest only in fresh seeds that were incubated at 22°C (Table S5). When seeds were imbibed at 10°C, all seeds germinated regardless of after-ripening duration, and when imbibed at 31°C, few seeds were able to germinate even after 5 months of after-ripening (Fig. S1). As on the L*er* background, therefore, an effect of *FLC* was only manifest under conditions of intermediate permissiveness for germination.

When *FRI* was non-functional, *FLC* disruption did not influence germination at any state of primary dormancy (Fig. 4; Table S5); even in fresh seeds incubated at 22°C, germination was similar with or without high *FLC*. As before, in the Col background, *FRI* activity appears to be necessary for *FLC* upregulation and consequent maximum germination.

*High-*FLC *activity is associated with reduced secondary dormancy induction (Experiment 2)*

On an active *FRI* background (Fig. 3; Table S5), the high *FLC* allele impeded secondary dormancy induction in response to hot stratification (significant Geno × Hot strat interaction in Table S3), although the magnitude of the effect varied with incubation temperature and after-ripening duration. In particular, in fresh seeds imbibed at 10°C, all seeds had high germination,

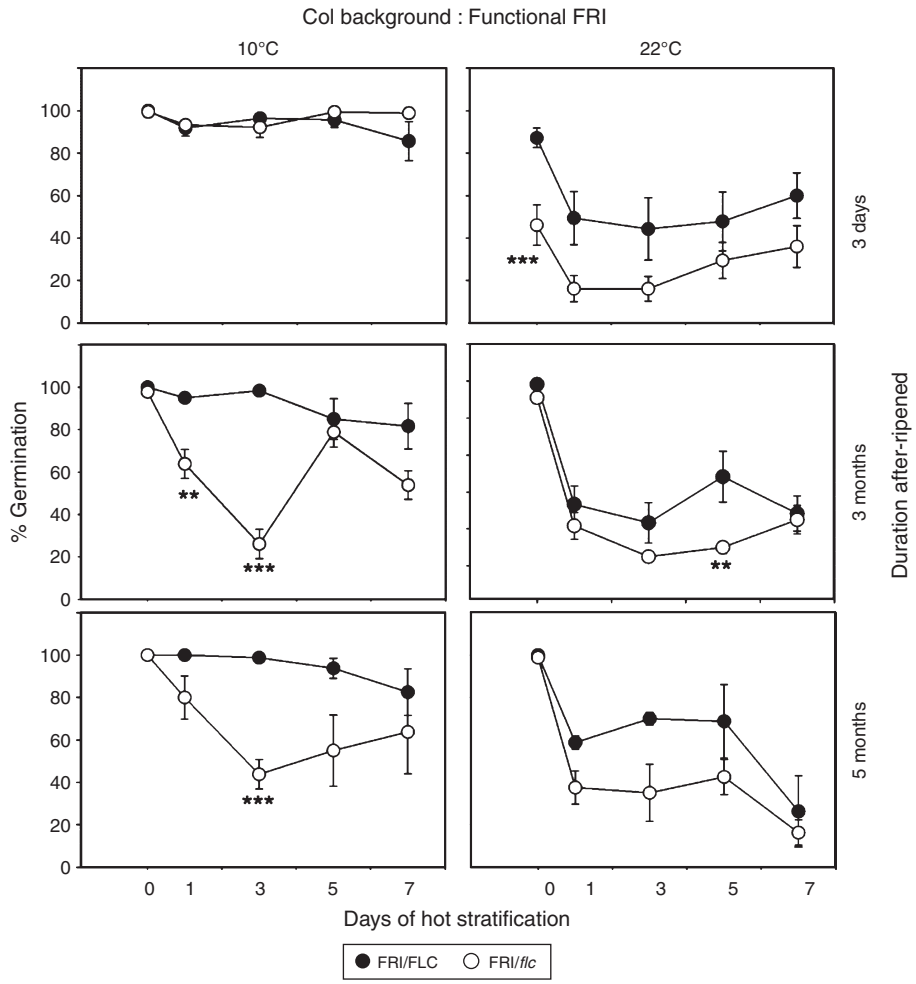

Fig. 3. Effect of *FLC* disruption over the course of after-ripening (Experiment 2) in the Col background, on a functional *FRI* background. Mean germination percentages (±s.e.) are shown for seeds after-ripened for 3 days, 3 months and 5 months, given different durations of hot stratification to induce secondary dormancy (*x*-axis), and imbibed at 10°C (left) and 22°C (right). Significant differences between genotypes are based on Wald Chi-square values from logistic regression as indicated: **, $P < 0.01$; ***, $P < 0.001$.

which masked differences between the genotypes. When *FRI* was inactive, disruption of *FLC* did not significantly influence germination in fresh seeds and those after-ripened for 3 months across all pre-incubation treatments (Fig. 4; Table S5). For seeds after-ripened for 5 months, the *FLC*-deficient genotype was induced into secondary dormancy by hot stratification (5 days), even when *FRI* was inactive. This suggests that *FLC* is capable of enhancing germination independently of *FRI*, provided seeds have lost sufficient primary dormancy.

The effect of FRI *on germination depends on* FLC *activity*

When *FRI* is disrupted on a functional *FLC* background, germination was reduced (*FRI*/*FLC* vs *fri*/*FLC*, black circles, in Fig. 3 c.f. Fig. 4, or see Figs S3 and S4 to see the same data plotted to contrast *FRI* genotypes; Tables 2, S2, S4, S5). This observation is consistent with the role of *FRI* in upregulating *FLC* and thereby promoting germination.

When *FLC* is non-functional, however, disruption of *FRI* was actually associated with higher germination in some treatments (*FRI*/*flc* vs *fri*/*flc* open circles, in Fig. 3 c.f. Fig. 4, or see Fig. S4 for the same data plotted to contrast *FRI* alleles; Tables S2, S4, S5). This effect was consistent across experiments (Fig. S2 – lower for Experiment 1; Fig. S4 for Experiment 2) and suggests that *FRI* may act independently of *FLC* to inhibit germination. This inhibitory effect of *FRI* in the absence of high *FLC* was apparent even in after-ripened seeds, although prolonged hot stratification (longer than 3 days) tended to mask the effect, since seeds even with non-functional *FRI* acquired dormancy.

Discussion

This experiment verifies that *FLC* enhances germination, since genotypes with higher *FLC* activity had higher germination than genotypes with lower *FLC* activity. Higher *FLC* activity not only reduced primary dormancy, causing higher germination of freshly shed seeds, but it also impeded the induction of secondary

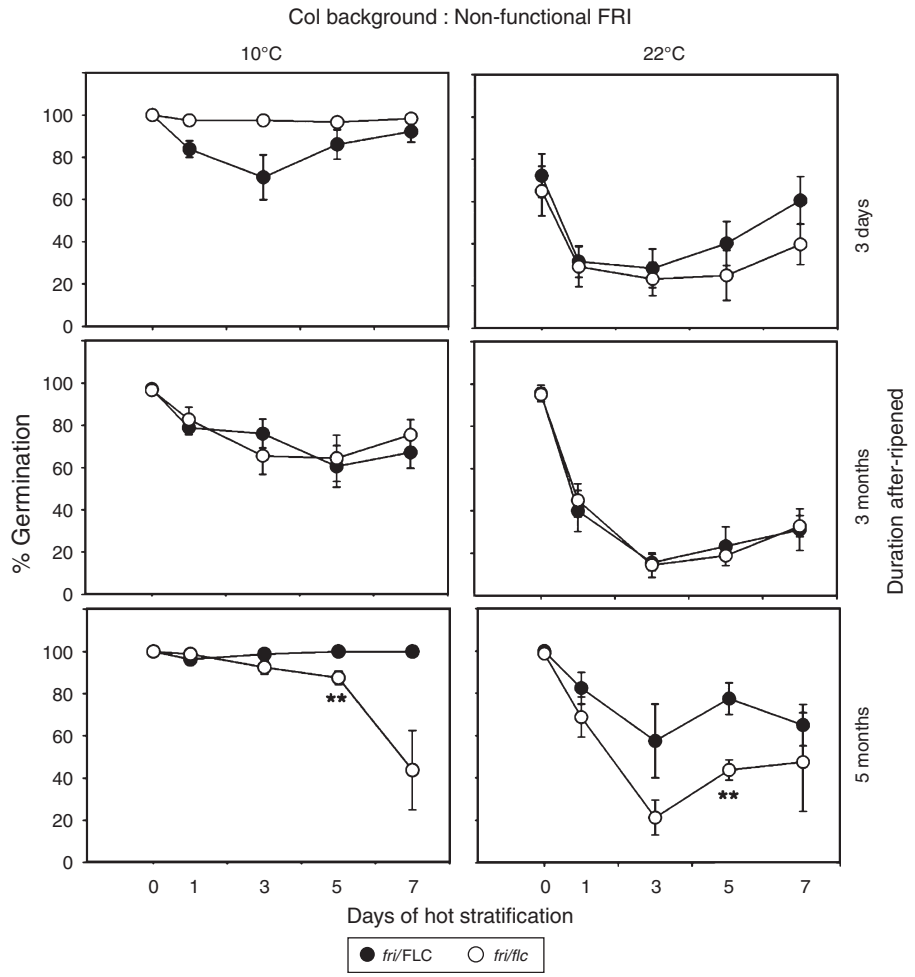

Fig. 4. Effect of *FLC* disruption over the course of after-ripening (Experiment 2) in the Col background, on a non-functional *FRI* background. Mean germination percentages (±s.e.) are shown for seeds after-ripened for 3 days, 3 months and 5 months given different durations of hot stratification induce secondary dormancy (*x*-axis), and imbibed at 10°C (left) and 22°C (right). Significant differences between genotypes are based on Wald Chi-square values from logistic regression as indicated: **, $P < 0.01$.

dormancy. The contribution of *FLC* to germination was manifest only at certain imbibition temperatures and stages of dormancy. Moreover, the positive regulator of *FLC* expression – *FRI* – also appears to be involved in germination via regulation of *FLC* expression and also potentially through a weaker pathway that is independent of *FLC* expression.

FLC *enhances germination of fresh seeds and impedes secondary dormancy induction*

In both genetic backgrounds (L*er* and Col), direct manipulation of *FLC* activity altered germination, such that increased *FLC* activity increased germination of fresh seeds and reduced the induction of secondary dormancy in response to hot stratification. This result is consistent with a previous study that demonstrated that a NIL (on the L*er* background) with high *FLC* and a transgenic line that overexpressed *FLC* (with 35S::*FLC* on the Col background) had higher germination than their low-*FLC* controls (Chiang *et al*. 2009). By specifically reducing *FLC* expression using RNAi, this study verified that the difference in germination between the NIL and its control (L*er*) background is caused at least in part by differences in *FLC* activity. By using mutants with disrupted *FLC* activity, this study verifies that mutational disruption of *FLC* on the Col background reduces germination, consistent with the prior observation that overexpression of *FLC* in Col increased germination (Chiang *et al*. 2009). Therefore, *FLC* is causally associated with higher germination.

This study also showed that higher *FLC* activity impeded secondary dormancy induction. This result is consistent with a study in buried seeds under natural field conditions that showed that *FLC* exhibits seasonal cycling that corresponds to seasonal cycles in dormancy (Footitt *et al*. 2011). The experiment presented here shows that when high *FLC* is present, secondary dormancy is reduced. Thus cycles of *FLC* expression under natural seasonal conditions are likely to mediate secondary dormancy cycling. Other genes that mediate primary dormancy, such as *DOG1*, have also been shown to regulate secondary

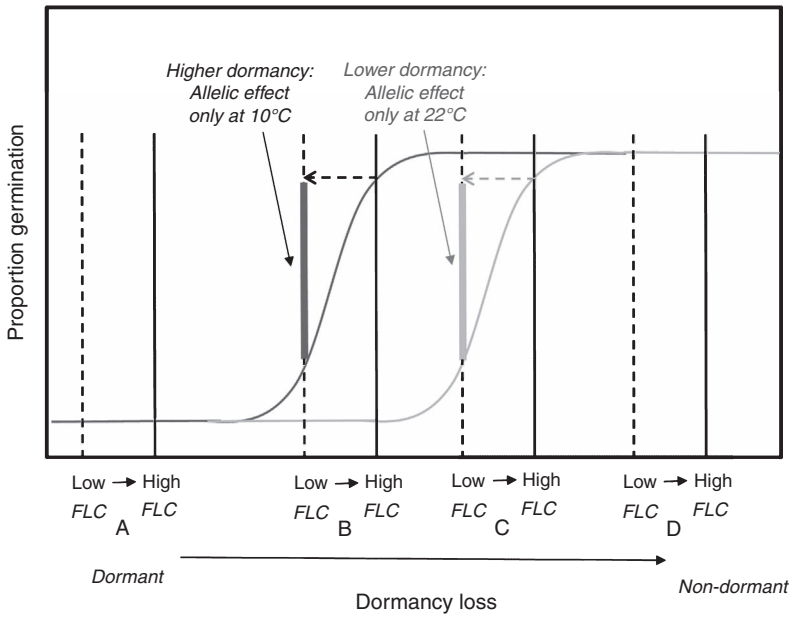

Fig. 5. Schematic of how temperature-dependent allelic effects change with dormancy status. The probability of germination (*y*-axis) increases as dormancy is lost (*x*-axis), via for example a longer duration of after-ripening. Germination can proceed first at low temperature (black/blue line), and as more dormancy is lost, germination can also proceed at higher temperature (grey/red line). High-*FLC* genotypes (solid vertical lines) have a greater ability to germinate than low-*FLC* genotypes (dashed vertical lines) under all conditions. (*a*) No allelic effects: when seeds are highly dormant, neither genotype can germinate at either temperature. (*b*) Large allelic effect at low temperature, but none at high temperature. With small amounts of dormancy loss, the high-*FLC* genotype germinates to high proportions at low temperature, while the low-*FLC* genotype germinates to lower proportions, but neither genotype germinates at high temperature. (*c*) Large allelic effect at high temperature, but none at low temperature. With larger amounts of dormancy loss, the high-*FLC* genotype germinates to high proportions at low and high temperature, while the low-*FLC* genotype germinates to high proportions only at low temperature, leading to no allelic effects at low temperature but large allelic effects at high temperature. (*d*) No allelic effect. Both genotypes germinate to high levels at low and high temperature. These dynamics of conditional dormancy (higher ability to germinate at a wider range of temperature as dormancy is lost) produce temperature-dependent allelic effects, and these temperature-dependent effects vary with underlying dormancy level.

dormancy (Footitt *et al*. 2011; Murphey *et al*. 2015), suggesting that primary and secondary dormancy share a genetic basis.

The contribution of FLC *to germination depends on temperature and dormancy*

In a previous study, the germination of genotypes with high *FLC* activity was enhanced much more strongly in seeds imbibed at 10°C than in seeds imbibed at 22°C (Chiang *et al*. 2009). Chiang *et al*. interpreted this result to indicate the *FLC* contributes to germination primarily at low temperature. While that interpretation applied to the conditions of their experiment, which were somewhat different from those used here, we found that the contribution of *FLC* to germination depends not on temperature *per se*, but on dormancy status and the conditions that promote germination; the influence of these combined factors can be manifest as temperature-dependent contributions of *FLC* to germination (Fig. 5).

First, in seeds with physiological dormancy, such as *A. thaliana*, dormancy is gradually lost through a process of after-ripening. Dormancy is therefore a continuous trait, such that the probability of germination increases with time of after-ripening. In this study, higher *FLC* activity caused a higher probability of germination over the course of after-ripening (compare solid and dotted lines in Fig. 5). However, in seeds with deep dormancy (far left in Fig. 5), high *FLC* activity alone is not adequate to elicit germination; other dormancy factors must be repressed or germination-enhancing factors increased. Likewise, in seeds with very low dormancy (far right in Fig. 5), even genotypes with low *FLC* activity were able to germinate. Thus effects of *FLC* on germination are manifest only at more intermediate levels of dormancy. This is because other genes also regulate dormancy and germination (reviewed in Holdsworth *et al*. 2008*b*).

Second, as physiological dormancy is lost, the range of conditions under which germination can occur widens (Fig. 5; Compare dark versus light grey curves: seeds are able to

germinate at higher temperature only when dormancy loss, is greater, via, for example, after-ripening); this is referred to as 'conditional dormancy' (Baskin and Baskin 2014). That is, seeds can exhibit intermediate levels of dormancy, such that they can germinate at some temperatures but not others, but they may acquire the ability to germinate at other temperatures as they continue to lose dormancy. Several *A. thaliana* genotypes, including the ones used in this experiment, first gain the ability to germinate at lower temperatures and subsequently, with further dormancy loss, gain the ability to germinate at higher temperature (Burghardt *et al*. 2016). In this experiment, this effect was manifest as higher germination at 10°C than at 22°C, especially in fresh seeds that have higher levels of primary dormancy, or in seeds induced into secondary dormancy. As seeds lose dormancy, they gain the ability to germinate even at the less permissive temperature of 22°C.

The combination of these dynamics leads to temperature-dependent contributions of *FLC* to germination that varies with dormancy status (Fig. 5). Specifically, when seeds have higher dormancy, the effect of *FLC* on germination is manifest only at the low (more permissive) temperature, but in seeds with lower dormancy, the effect of *FLC* on germination is manifest only at the higher (less permissive) temperature (bold coloured bars in Fig. 5). These dynamics can explain the difference between the results of Chiang *et al*. (Chiang *et al*. 2009), who used more dormant seeds and found *FLC* effects on germination primarily at low temperature, and this experiment, which used less dormant seeds and found *FLC* effects on germination to be more pronounced at higher temperature. In short, dormancy level influences both temperature-dependent germination and allelic effects of germination genes; this causes temperature-dependent allelic effects that change as dormancy is lost or gained.

These condition-dependent effects of *FLC* on germination have ecological significance. Differences among *FLC* genotypes are likely to be most pronounced when fresh seeds are shed at low temperature (but not warm temperature), leading to faster germination of high-*FLC* genotypes than low-*FLC* genotypes under those conditions. Likewise, after a period of after-ripening, low-*FLC* genotypes are likely to have slower germination than high-*FLC* genotypes only if temperatures stay warm, but not if temperatures drop. Therefore, natural selection on *FLC* alleles, via their effects on germination, is expected to depend on intrinsic dormancy levels influenced by other dormancy genes, the temperature at the time of seed shed, and the temperature after a period of after-ripening. Within the context of the typical winter-annual life history exhibited by *A. thaliana*, in which seeds are shed in warm temperatures of spring, *FLC* is expected to accelerate germination in the autumn – allowing germination at slightly higher temperature than would be necessary for germination of low-*FLC* genotypes. Moreover, in the more unusual autumn-flowering life history, high-*FLC* genotypes may even be able to germinate that same autumn in which the plants flower, when fresh seeds are shed at lower temperature. Thus high-*FLC* activity appears to promote (earlier) germination in autumn under natural seasonal conditions. This prediction accords with a field study that showed that high-*FLC* genotypes did have more germination in the autumn season than did low-*FLC* genotypes (Chiang *et al*. 2009).

FRI *regulates germination*

The positive regulator of *FLC*, *FRI*, also influenced germination in this experiment. First, the Col allele of *FLC* required active *FRI* in order to influence germination; disruption of *FLC* only influenced germination on a functional *FRI* background. Because the Col allele of *FRI* is naturally inactive, this result is consistent with the lack of differences in germination between the Col background and the *flc-3* mutation observed by Liu *et al*. (2011). That is, *FLC* must be upregulated by *FRI* before its disruption can influence germination. It should be noted that in the L*er* background, the effect of *FLC* was not contingent on *FRI* activity (L*er* naturally has non-functional *FRI*), but this is because the specific allele that was used from the Cape Verde ecotype does not require *FRI* for *FLC* to be expressed (Gazzani *et al*. 2003). Second, the strongest effect of *FRI* occurred on a functional *FLC* background, such that disruption of *FRI* caused lower germination. This effect is consistent with the hypothesis that *FRI* enhances germination by upregulating *FLC*, in the same manner that *FRI* upregulates *FLC* at the pre-reproductive stage (Michaels and Amasino 2001; Caicedo *et al*. 2004; Michaels *et al*. 2004; Stinchcombe *et al*. 2004; Le Corre 2005; Shindo *et al*. 2005, 2006). In addition to *FLC* being expressed at the pre-reproductive stage, Chiang *et al*. (Chiang *et al*. 2009) found that *FLC* is also expressed at high levels late in silique development, at the time that dormancy is typically induced, suggesting that *FLC* expression in siliques regulates germination. Further experiments are necessary to test whether *FLC* activity during silique development depends on *FRI*.

We note that in the Col background, *FRI* appeared to exhibit a secondary antagonistic effect on germination independently of *FLC*; when *FLC* was not functional, *FRI* disruption actually increased germination slightly. Although this effect was quite small, it was apparent in multiple experiments and further suggests that *FRI* is involved in the regulation of seed germination. Thus, not only is *FLC* – a major flowering regulator – involved in germination, but other components of the *FLC*-mediated flowering pathway are also involved in germination. Pleiotropy of life-history genes such as *FLC* and *FRI* is important within the context of adaptive life cycles. First, if an allelic change in a gene causes changes in more than one life stage, those changes may be favourable for one trait but unfavourable for the other; if so, pleiotropy may constrain the expression of adaptive life histories. In contrast, if an allelic change leads to a favourable change in multiple, integrated phenotypes, then such pleiotropy may be adaptive. In the case of *FLC* (mediated by *FRI*), high *FLC* expression promotes germination in the autumn (as explained above), and also suppresses flowering until spring, leading to a winter-annual life history. In this case, allelic changes that alter *FLC* activity at both life stages could fundamentally alter the life history that is expressed.

Despite the fact that *FLC* and *FRI* were first identified as flowering regulators, the fact that they also influence germination implies that their evolutionary history was determined not only by natural selection on flowering time, but also by natural selection on germination phenology. Germination time is known to be under strong natural selection in *A. thaliana* (Donohue *et al*. 2005; Huang *et al*. 2010), and germination and dormancy have been shown to contribute to local adaptation of *A. thaliana*

(Kronholm *et al*. 2012; Montesinos-Navarro *et al*. 2012). Therefore, in addition to flowering time, the effects of *FLC* and *FRI* on germination may also strongly contribute to the geographic distribution of genetic variation of these genes (Caicedo *et al*. 2004; Stinchcombe *et al*. 2004, 2005).

Conclusion

A major flowering gene, *FLC*, and one of its primary regulators, *FRI*, both contribute to germination. A compelling question for future research is the extent to which other genes involved in *FLC* regulation, and other genes regulated by *FLC*, are also involved in germination. Moreover, these genes may influence germination via their interactions with *FLC*, or independently of it. How concordant these genetic pathways of flowering and germination regulation are would influence the strength of pleiotropy across life stages, influence the expression of coordinated life histories, and determine patterns of natural selection on major life-history loci.

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