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The autonomous flowering-time pathway pleiotropically regulates seed germination in *Arabidopsis thaliana*

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• **Background and Aims** Two critical developmental transitions in plants are seed germination and flowering, and the timing of these transitions has strong fitness consequences. How genetically independent the regulation of these transitions is can influence the expression of life cycles.
• **Method** This study tested whether genes in the autonomous flowering-time pathway pleiotropically regulate flowering time and seed germination in the genetic model *Arabidopsis thaliana*, and tested whether the interactions among those genes are concordant between flowering and germination stages.
• **Key Results** Several autonomous-pathway genes promote flowering and impede germination. Moreover, the interactions among those genes were highly concordant between the regulation of flowering and germination.
• **Conclusions** Despite some degree of functional divergence between the regulation of flowering and germination by autonomous-pathway genes, the autonomous pathway is highly functionally conserved across life stages. Therefore, genes in the autonomous flowering-time pathway are likely to contribute to genetic correlations between flowering and seed germination, possibly contributing to the winter-annual life history.

Key words: *Arabidopsis thaliana*, autonomous pathway, flowering time, genetic pathway, germination, phenotypic plasticity, pleiotropy

INTRODUCTION

Two critical developmental transitions in plants are seed germination and flowering. The timing of both transitions can be subject to strong natural selection; the timing of seed germination determines the seasonal conditions to which seedlings and later life stages are exposed (reviewed by Donohue *et al.*, 2010; Postma *et al.*, 2016), and the timing of reproduction determines the duration of seasonal conditions appropriate for reproduction (Munguia-Rosas *et al.*, 2011; Ehrlen, 2015). Therefore, each developmental transition needs to be timed accurately to maximize fitness.

How genetically independent these distinct developmental transitions are could influence the expression of optimal life cycles, since each transition needs to respond optimally to its own internal and environmental cues. It is therefore important to know whether common genetic pathways pleiotropically regulate both flowering and germination. On the one hand, pleiotropy may facilitate the evolution of coordinated responses of multiple functionally related phenotypes; on the other, pleiotropy may prevent optimum phenotypes from evolving for any single trait (Fisher, 1958; Atchley, 1984; Wagner, 1988; Barton, 1990; Crespi, 2000; Orr, 2000; Griswold and Whitlock, 2003; Brakefield, 2006; Wagner *et al.*, 2008; Walsh and Blows, 2009). Pleiotropy moreover is expected to be strongest when traits share entire genetic pathways of regulation. It is therefore important to know not only whether common genes regulate multiple developmental transitions, but also whether they do so through shared genetic pathways.

In the model genetic organism, *Arabidopsis thaliana*, pleiotropy has been documented in the regulation of flowering and germination. Specifically, the gene *FLOWERING LOCUS C* (*FLC*) was first identified as a major regulator of flowering time (Michaels and Amasino, 1999), integrating cues from the vernalization and autonomous flowering pathways (Simpson and Dean, 2002; Michaels *et al.*, 2005; Ream *et al.*, 2012). *FLC* prevents flowering by repressing the floral integrators *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*), *FLOWERING LOCUS T* (*FT*) and FLOWERING LOCUS D (*FD*). *FLC* has since been shown to influence germination, with high-*FLC* genotypes showing more germination (Chiang *et al.*, 2009; Blair *et al.*, 2017).

More recently, regulators of *FLC* expression have also been shown to have pleiotropic effects on seed germination. Genes in the flowering-vernalization pathway regulate *FLC* expression at the vegetative stage in response to winter chilling. *FRIGIDA* (*FRI*) and *VERNALIZATION INDEPENDENCE3* (*VIP3*) upregulate *FLC* (Michaels and Amasino, 2001; Zhang *et al.*, 2003; Amasino, 2004; Dennis and Peacock, 2007; Choi *et al.*, 2009; Ream *et al.*, 2012), while *VERNALIZATION INSENSITIVE3* (*VIN3*) and *VERNALIZATION2* (*VRN2*) epigenetically silence *FLC* in response to chilling (Sung and Amasino, 2006; Ream

et al., 2012). All those genes have also been shown to influence seed germination (Auge *et al.*, 2017). Some did so in a manner that is expected based on their function as *FLC* repressors during the vegetative phase, but some appeared to have a degree of functional divergence between the vegetative stage, which determines flowering time, and the seed-maturation stage, which determines seed dormancy.

This study tests whether genes in a different flowering pathway – the autonomous flowering pathway – influence seed germination. Genes in the autonomous flowering pathway – *FCA*, *FPA*, *FY*, *FLK*, *FVE*, *FLD*, *LD* and others (Koornneef *et al.*, 1991, 1998; Lee *et al.*, 1994) – interact indirectly with the other flowering pathways to regulate flowering, by repressing *FLC* expression and thereby promoting flowering (Michaels and Amasino, 2001; Reeves and Coupland, 2001; Rouse *et al.*, 2002; Lim *et al.*, 2004; Mockler *et al.*, 2004; Chen *et al.*, 2005; Bäurle and Dean, 2008; Lee and Amasino, 2013). These genes are not directly involved in flowering responses to photoperiod or vernalization, but they regulate flowering responses to ambient temperature, allowing plants to flower more quickly especially under higher temperature (Blázquez *et al.*, 2003). The genes in the autonomous pathway collectively regulate *FLC* mRNA levels via various epigenetic processes. *FCA*, *FPA* and *FLK* have RNA binding sites and are involved in RNA processing (Macknight *et al.*, 1997; Schomburg *et al.*, 2001; Simpson *et al.*, 2003; Lim *et al.*, 2004). Although *FLK* acts independently of *FCA* (Ripoll *et al.*, 2009), other genes interact through various epigenetic mechanisms. *FPA* and *FCA* (interacting with *FY*; Simpson *et al.*, 2003; Yu and Michaels, 2010) both have negative autoregulation via the polyadenylation and cleavage of their own RNA (Quesada *et al.*, 2003; Xing *et al.*, 2008; Hornyik *et al.*, 2010). In addition, they are involved in the polyadenylation of antisense *FLC*, which then interacts with *FLD* to demethylate histones associated with *FLC*, resulting in the downregulation of *FLC* (Liu *et al.*, 2007, 2010; Yu and Michaels, 2010). *FVE* is also involved in the epigenetic regulation of *FLC*, via histone methylation, and both *FLD* and *FVE* are involved in *FLC*-associated histone deacetylation (He *et al.*, 2003; Ausín *et al.*, 2004; Bäurle *et al.*, 2007; Bäurle and Dean, 2008; Pazhouhandeh *et al.*, 2011; Yu *et al.*, 2016). Thus, the genes interact via epigenetic interactions to regulate *FLC* levels and thereby flowering (Koornneef *et al.*, 1998; Simpson, 2004).

One gene in the autonomous flowering pathway – namely *FY* – has been studied within the context of seed germination (Jiang *et al.*, 2012; Cyrek *et al.*, 2016). Specifically, disruption of *FY* was shown to increase germination by decreasing sensitivity to the dormancy-inducing hormone abscisic acid (ABA). Moreover, *FY* has been shown to be involved RNA 3′ processing and to regulate the alternative polyadenylation of transcripts of the major dormancy regulator, *DELAY OF GERMINATION-1* (*DOG1*: Bentsink *et al.*, 2006). *FY* appears to be involved in the production of the biologically active short isoform transcript of *DOG1* (Cyrek *et al.*, 2016). Although this individual autonomous-pathway gene, *FY*, has been implicated in seed germination, whether the interactions among genes in that pathway are also preserved across flowering and germination remains unknown.

This study investigates pleiotropy of flowering-time autonomous-pathway genes across two major developmental transitions in plants: flowering and seed germination. The following were investigated: (1) Does disruption of autonomous-pathway genes increase seed germination, consistent with their role of being repressors of *FLC*? (2) Are the interactions among autonomous-pathway genes consistent between the major developmental transitions of flowering and germination?

MATERIALS AND METHODS

Genetic material and plant growth conditions

Mutations in the flowering-time autonomous pathway were used to compare their effects on flowering time and germination. All mutants were on the Landsberg *erecta* (L*er*) reference ('wild type') genetic background from which the mutants were derived. Mutants include single knockout/knockdown mutants of the genes *FLK*, *FCA*, *FPA*, *FVE* and *FY*, as well as pairwise combinations of those mutants (*fpa*/*fca*, *fve*/*fca*, *fve*/*fpa*, *fy*/*fca*, *fy*/*fpa*, *fy*/*fve)*. Supplementary Data Table S1 provides a list of mutants and ABRC stock numbers.

All genotypes were grown in a common environment to provide seeds for the experiment, as follows. Seeds were stratified at 4 °C for 7 d on 0.6 % (w/v) agar to induce germination, and they were then transferred to pots and allowed to germinate in a 12-h light/12-h dark cycle at 22 °C. After 1 week, seedlings were thinned and grown until seeds were harvested. These seeds were used to generate the maternal generation of this experiment. The maternal generation was grown as follows. Because of differences in flowering speed among the genotypes, slower flowering genotypes were planted before faster flowering genotypes; this schedule resulted in synchronized seed maturation across all genotypes for production of the seeds used in the germination assays described below. Seeds of each genotype were sown in 0.6 % (w/v) agar plates, stratified at 4 °C for 7 d in darkness, then transferred to pots with potting soil (Metromix 360 soil, Scotts Sierra, Marysville, OH, USA) in EGC GCW-30 Plant Growth Chambers (Environmental Growth Chambers, Chagrin Falls, OH, USA) under a 12-h light/12-h dark cycle [photosynthetically active radiation (PAR): 290 µmol m^{-2} s^{-1}] at 22 °C to induce germination. Twelve replicate pots per genotype, thinned to one seedling per pot, were kept in the same conditions until bolting (when inflorescence stems were approximately 1 cm tall). All plants bolted within 10 d of one another. All plants were then transferred simultaneously to short day (8 h light/16 h darkness) at 15 °C constant temperature and were then fertilized once with a 300 p.p.m. nitrogen solution of Blossom Booster Fertilizer (JR Peters, Allentown, PA, USA). Plants were monitored approximately every 3 d. The timing of bolting (transitions from vegetative to reproductive state: 'bolting date') and the number of rosette leaves present at the time of bolting ('leaf number') were recorded. The number of leaves indicates the developmental stage at which bolting occurred. After at least 75 % of the siliques on all plants were mature, watering was withheld 1 week before harvest to induce the drying of siliques. Harvest of all plants occurred on the same day, after siliques had dried. After harvesting, all seeds were kept in dry storage (Secador® 4.0 Auto-Desiccator Cabinets, Bel-Art Products, Pequannock, NJ, USA) at room temperature. 'Fresh' seeds were used in germination assays 3 d after harvest. 'After-ripened' seeds were kept at room temperature in a desiccator cabinet for 3 months.

Germination assays

Germination of fresh and after-ripened seeds was assayed at two temperatures: 10 and 22 °C. Seeds were sown in 35-mm Petri plates with 0.6 % (w/v) agar and immediately incubated at 10 or 22 °C in a 12-h light/12-h dark cycle (PAR: 120–150 µmol m^{-2} s^{-1}) in EGC Model GC8-2 Plant Growth Chambers (Environmental Growth Chambers). Twelve replicates (plates) were plated, for each treatment and genotype, with 20 seeds per plate (12 replicates × 12 genotypes × 2 temperatures × 2 after-ripening durations = 576 plates total). 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 21 d after the beginning of incubation in the light, after germination had reached a clear plateau (usually after 14 d).

Statistical analysis

'Bolting date' was calculated as the number of days between seed sowing and the time of bolting, and it was natural-log transformed to normality; leaf number at the time of bolting was normally distributed. These two metrics of flowering time were analysed with analysis of variance (ANOVA), using PROC GLM in SAS 9.4 (SAS Institute, Cary, NC, USA) to test for significant effects of the mutations. Bolting date or leaf number were the dependent variables, and genotype was a fixed effect. Bolting date and leaf number were strongly correlated (Pearson's correlation = 0.74, $P < 0.001$), and results were similar for both metrics of flowering time, so the discussion is restricted to the analysis of leaf number. Supplemental Data Fig. S1 and Table S2 provide analysis of bolting date. Comparisons of each mutant genotype were made to the L*er* wild type background using the 'contrast' statement. Comparisons of double mutants to their corresponding single mutants were made in sub-models that included only L*er*, the two single mutants and the double mutant, and the single mutants were compared to the double mutant. Significance levels were Bonferroni-corrected for multiple comparisons. To test for significant interactions between allelic states for each double-mutant group (deviation from additive effects of each locus), all genotypes were scored as functional or non-functional at each of the genotype's two loci ('Locus 1' refers to the first gene indicated in the genotype name, and 'Locus 2' refers to the second gene indicated in the genotype genotype name, for each double mutant); ANOVA tested for a significant interaction between Locus 1 and Locus 2 in a model that included each locus and their interaction.

The final proportion of seeds that germinated was analysed with logistic regression (PROC GENMOD in SAS 9.4; SAS Institute) using a binomial distribution and a logit link function, to perform likelihood ratio tests based on Wald chi-square. First, a full model was used to test for higher-level interactions. Germination proportion was the dependent variable, and genotype, temperature treatment and after-ripening treatment were fixed effects. Because of highly significant interactions between genotype and temperature and between genotype and after-ripening, we tested for significant differences among genotypes within each after-ripening and temperature treatment separately. Each genotype was compared to the L*er* wild type background in a single model; comparisons of double mutants to each of their component single mutants were conducted as described above, with the double mutant as the reference genotype, and significance levels were Bonferroni-corrected for multiple comparisons. To test whether mutational effects differed significantly across temperature, we tested for significant Genotype × Temperature interactions in fresh and after-ripened seeds separately in a model that included all genotypes (with the L*er* wild type as the reference genotype). Likewise, to test whether allelic effects differed significantly across after-ripening treatments, we tested for significant Genotype × After-ripening interactions for each temperature separately.

RESULTS

Mutational disruption of each gene in the autonomous flowering-time pathway studied here resulted in significantly delayed flowering time, as expected (Table 1, Fig 1, Supplementary Data Table S2 and Fig. S1). Disruption of many of those genes also altered germination proportions in a manner that depended on temperature (Genotype × temperature: Wald's chi-square = 79.61, $P < 0.001$) and after-ripening (Genotype × After-ripening: Wald's chi-square = 38.46, $P < 0.001$). After-ripened seeds lost dormancy and therefore had higher germination proportions than fresh seeds, as expected (After-ripening: Wald's chi-square = 197.46, $P < 0.001$).

Regarding single mutants, disruption of *FLK*, *FPA* (after-ripened seeds only), *FVE* and *FY* individually delayed flowering and increased germination at 10 °C (Figs 1 and S2A, Tables 1, 2, S2 and S3). At 22 °C, disruption of *FY* increased germination of fresh seeds, while disruption of *FPA* decreased germination at 22 °C in after-ripened seeds, revealing a temperature-dependent effect of *FPA* disruption on germination.

Interactions among the genes were evaluated by quantifying the effects of disrupting each pair of autonomous-pathway genes. Disruption of *FCA* and *FPA* (Figs 1 and S2B, Tables 1, 2, S2 and S3) contributed additively to the delay of flowering time, with

TABLE 1. *Effects of mutation on flowering time*

Locus 1	Locus 2	Double vs. Locus 1	Double vs. Locus 2	Locus 1 × Locus 2
fpa	*fca*	12.81***	16.07***	3.39
fve	*fca*	0.46	15.60***	30.23***
fve	*fpa*	12.57**	1.32	76.55***
fy	*fca*	3.21	0.34	11.11**
fy	*fpa*	0.08	2.10	26.60***
fy	*fve*	4.99	5.92	28.53***

F-values are given, based on ANOVA to test for effects of mutation on the number of leaves at the time of bolting, a metric of flowering time. 'Locus 1' is listed in the first column, and 'Locus 2' is listed in the second column; double mutants have non-functional alleles at both loci. 'Double vs. Locus 1' and 'Double vs. Locus 2' compare the phenotype of the double mutant to the phenotype of each single mutant. 'Locus 1 × Locus 2' tests for significant interactions (non-additivity) between allelic states (functional vs. non-functional) of the two loci. $^{**}P < 0.01$, $^{***}P < 0.001$. Any asterisk indicates significance after Bonferroni correction. See Supplementary Data Table S2 for comparisons of each genotype to the L*er* wild type and for analysis of bolting time.

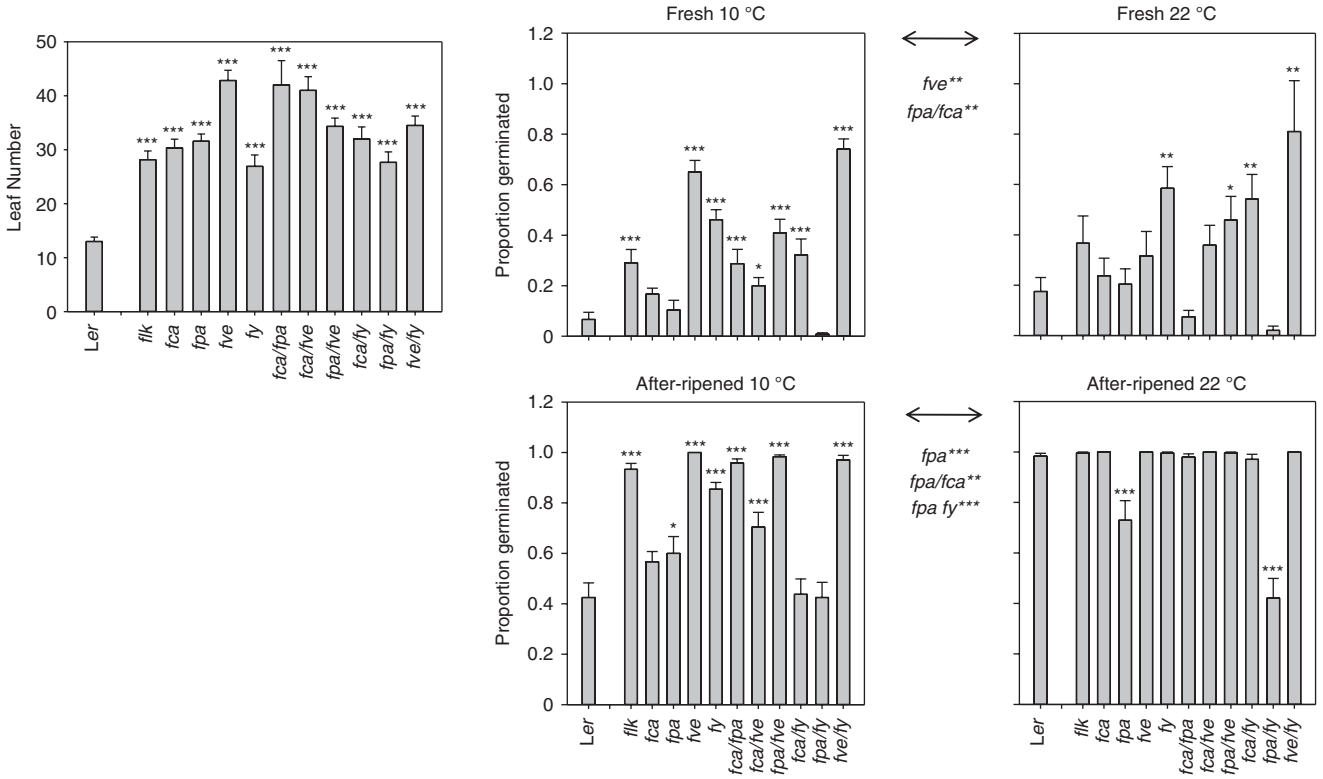

FIG. 1. Differences between mutant genotypes and the L*er* wild type in flowering time and germination. Mean (+s.e.) leaf number at the time of bolting, a metric of flowering time (upper left), and germination proportion of fresh (upper) and after-ripened (lower) seeds incubated at 10 °C (middle) and 22 °C (right). Asterisks indicate significant differences compared to the L*er* wild type background. Arrows between panels indicate significance differences in mutational effects (comparison to L*er*) between temperature, with the genotype that varies indicated below the arrow. See Table 2 and Supplementary Data Fig. S2 for comparisons of single and double mutants. $*P < 0.017$ (Bonferroni threshold); $**P < 0.01$; $***P < 0.001$.

TABLE 2. *Comparison of germination proportions between single and double mutants*

Locus 1	Locus 2	Double vs. Locus 1	Double vs. Locus 2	Locus 1 × Locus 2	Double vs. Locus 1	Double vs. Locus 2	Locus 1 × Locus 2
Fresh			10 °C			22 °C	
fpa	*fca*	8.37**	3.44	0.10	3.11	4.37	3.63
fve	*fca*	48.08***	0.47	38.15***	0.13	1.10	0.05
fve	*fpa*	9.16**	17.43***	5.09*	1.21	4.04	0.23
fy	*fca*	4.06	6.32	8.81**	0.07	6.41*	0.41
fy	*fpa*	19.65***	6.01	40.63***	16.42***	5.66	23.82***
fy	*fve*	16.34***	1.92	17.80***	0.36	3.79	0.07
After-ripened		10 °C			22 °C		
fpa	*fca*	21.17***	23.43***	13.04***	13.40***	0.00	0.16
fve	*fca*	0.00	4.10	47.69***	0.00	0.00	0.00
fve	*fpa*	0.00	21.71***	3.79	0.00	7.94**	0.04
fy	*fca*	30.18***	2.97	32.18***	3.22	0.00	9.91***
fy	*fpa*	24.85***	4.26	29.11***	7.77**	10.62***	1.78
fy	*fve*	9.42**	0.00	16.28***	–	0.00	0.00

Wald chi-square values are given, based on logistic regression. Results are provided separately for each temperature (10 and 22 °C) and for each after-ripening treatment (Fresh = 3 d after-ripened; After-ripened = 3 months after-ripened). Column headings are the same as in Table 1. See Supplementary Data Table S3 for comparisons of each genotype to the L*er* wild type. '–' indicates contrast could not be computed because of lack of variance. $*P < 0.017$ (Bonferroni threshold value), $**P < 0.01$, $***P < 0.001$.

the double mutant having more delayed flowering than either single mutant. When seeds were incubated at 10 °C, disruption of *FPA* alone increased germination (after-ripened seeds). The double mutant had a significantly higher germination proportion than the single *fpa* (both fresh and after-ripened seeds) and the *fca* (after-ripened seeds) single mutant, suggesting the contribution of both loci to the repression of germination.

Disruption of *FCA* and *FY* each delayed flowering, but the double mutant had a flowering time that was not significantly different from that of either single mutant (Figs 1 and S2C,

Tables 1, 2, S2 and S3). This pattern suggests that *FCA* and *FY* interact to promote flowering, as has been documented previously (Simpson *et al.*, 2003; Yu and Michaels, 2010). These genes also interacted to influence germination. The *fca* single mutant did not differ from the L*er* wild type in any treatment. In fresh seeds, the single *fy* and the *fca/fy* double mutant had comparably higher germination than L*er*. This result suggests that *FCA* does not influence germination in fresh seeds, alone or through its interactions with *FY*, but that functional *FY* inhibits germination. In after-ripened seeds at 10 °C, an interesting pattern was found that does implicate *FCA* in germination: disruption of *FY* alone enhanced germination, but the *fca/fy* double mutant had lower germination than the *fy* single mutant, and comparable germination to L*er* and the *fca* single mutant. Therefore, the effect of disrupting *FY* was only apparent when *FCA* was functional. This pattern suggests that functional *FCA* can enhance germination when *FY* is inactive (Fig. 2A and B). After-ripened seeds of all genotypes germinated to 100 % at 22 °C.

Disruption of *FCA* and *FVE* both caused a delay of flowering, but disruption of both genes resulted in the same flowering behaviour as disruption of *FVE* alone (Figs 1 and S2D, Tables 1, 2, S2 and S3). This pattern suggests that the effect of *FCA* on flowering can be explained by its interaction with or regulation of *FVE*. After-ripened seeds incubated at 10 °C showed a similar pattern: disruption of *FVE* and concurrent disruption of both *FVE* and *FCA* resulted in increased germination. For fresh seeds at 10 °C, however, disruption of *FVE* caused an increase in germination, but the double mutant had significantly less germination than the single *fve* mutant but similar germination to the L*er* wild type and the single *fca* mutant. This pattern suggests that *FCA* may enhance germination when *FVE* is not active (Fig. 2A and B). At 22 °C, disruption of these genes did not significantly alter germination.

Disruption of *FPA* and *FY* each delayed flowering, but the double mutant had a flowering time that was not significantly different from that of either single mutant (Figs 1 and S2E, Tables 1, 2, S2 and S3). This pattern suggests that *FPA* and *FY* interact to promote flowering. For seeds incubated at 10 °C, disruption of *FY* alone enhanced germination, but the *fpa/fy* double mutant had significantly lower germination than the *fy* single mutant, but did not differ from the wild type or the *fpa* single mutant. Therefore, *FPA*, like *FCA*, may enhance germination when *FY* is inactive (Fig. 2A and B). This same pattern was observed at 22 °C, except that in after-ripened seeds, even the *fpa* single mutant had lower germination than the L*er* wild type, suggesting that this gene can enhance germination under some conditions, even when *FY* is active.

Disruption of *FPA* and *FVE* each delayed flowering, but the double mutant had a flowering time that was significantly earlier than the single *fve* mutant (Figs 1 and S2F, Tables 1, 2, S2 and S3). This pattern suggests that *FPA* promotes flowering via its interaction with *FVE*, but that when *FVE* is not active it may delay flowering. Germination at 10 °C resembled this pattern, but not completely. In fresh seeds, disruption of *FPA* alone did not alter germination, although the double mutant showed significantly less germination than the *fve* single mutant. This pattern suggests that, like *FCA*, *FPA* enhances germination when *FVE* is not active (Fig. 2A and B). In after-ripened seeds at 10 °C, disruption of each gene enhanced germination, but the double mutant had the same germination as the *fve* single mutant, suggesting either additive contributions that resulted in maximal germination, such that any germination-enhancing contribution of *FPA* (reduction in germination caused by *FPA* disruption) is no longer detectable in highly after-ripened seeds, or that *FPA* represses germination solely through its interaction with *FVE*. At 22 °C, disruption of *FVE* appears to increase germination in fresh seeds, since the double mutant had significantly higher germination than the L*er* wild type, and the *fve* mutant did not differ significantly from the double mutant in its germination. In after-ripened seeds at 22 °C, disruption of *FPA* alone resulted in less germination, suggesting functional *FPA* can enhance germination at 22 °C (as discussed above).

Disruption of *FVE* and *FY* each delayed flowering, but the double mutant had a flowering time that was not significantly different from that of either single mutant (Figs 1 and S2G, Tables 1, 2, S2 and S3). This suggests that *FVE* and *FY* interact to promote flowering. For seeds incubated at 10 °C, disruption of each gene alone increased germination, but the double mutant had a germination proportion that was not significantly different from the *fve* single mutant. This pattern suggests that functional *FY* inhibits germination through its interaction with *FVE*. At 22 °C, *fy* and the double mutant had comparably higher germination than the L*er* wild type (fresh seeds), indicating that functional *FY* impedes germination at 22 °C. All after-ripened seeds germinated to high proportions at this temperature.

DISCUSSION

Several genes in the autonomous flowering-time pathway pleiotropically influenced seed germination. The majority of these genes acted to promote flowering and impede germination, but their effect on seed germination sometimes varied with temperature or degree of after-ripening. Some genes interacted with each other in their regulation of both flowering time and germination, sometimes synergistically but sometimes in opposition to one another. The pattern of these interactions was similar between the regulation of flowering and the regulation of germination, especially at 10 °C, suggesting the conservation of gene function across these two developmental transitions. However, we did find evidence that some genes in the autonomous pathway may promote germination, which is not predicted based on their known function in *FLC* repression during the transition to flowering.

All the genes studied here promoted flowering, consistent with their role of repressing *FLC*. Only *FPA* appeared to have some antagonistic effect on flowering in its interaction with *FVE*; although *FPA* appears to promote flowering when *FVE* is functional, it also appears to have an antagonistic effect that is apparent in the absence of functional *FVE*, such that the double mutant *fpa/fve* had earlier flowering than the single *fve* mutant. *FPA* also appeared to interact non-additively with *FY*, *FCA* interacted with *FVE* and *FY*, and *FY* and *FVE* interacted with each other in the regulation of flowering. Several of these interactions accord with known interactions among these genes (Koornneef *et al.*, 1998; Simpson *et al.*, 2003; Simpson, 2004; Liu *et al.*, 2007, 2010; Yu and Michaels, 2010). Although we detected some non-additive interactions that have not always been observed (specifically the potentially antagonistic effect of *FPA* in the absence of functional *FVE*; Bäurle *et al.*, 2007), the positive contribution of these genes to flowering is consistent with current knowledge of their function as repressors of *FLC*.

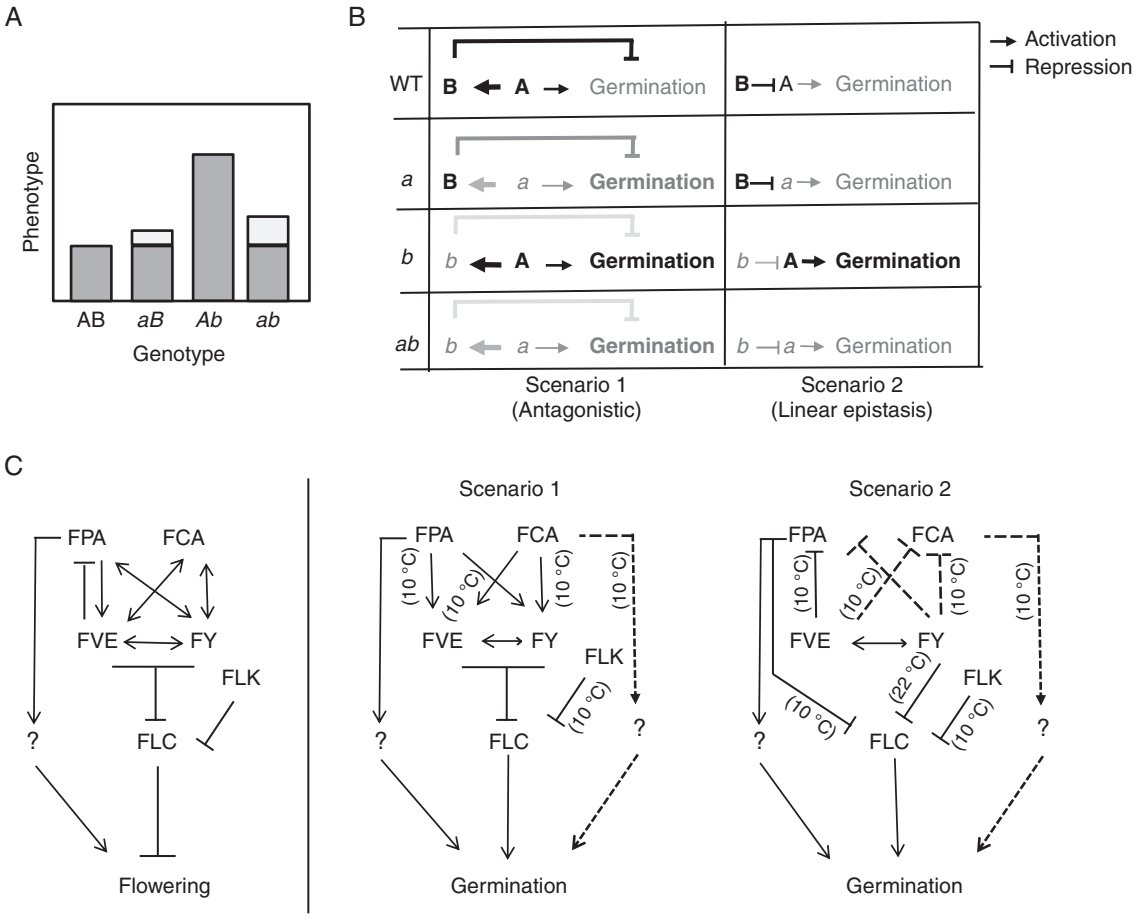

FIG. 2. Inferred genetic pathway whereby genes in the autonomous flowering-time pathway influence flowering and germination, via the expression of *FLC*, which represses flowering and increases germination. (A) A recurrent pattern observed in single and double mutants, which is not predicted from the function of autonomous-pathway genes being repressors of *FLC*. Capital letters (A and B) indicate wild type alleles at locus A and locus B, respectively, and lower case letters (a and b) indicate mutant alleles at those loci. 'Phenotype' refers to germination or flowering time. (B) Two genetic pathways that can account for the pattern observed in panel A. Scenario 1 shows two antagonistic pathways, with the repressing pathway stronger than the promoting pathway. Scenario 2 shows a linear epistatic pathway whereby one gene represses the other, which in turn promotes germination. Scenario 2 produces the pattern depicted by the dark grey bars in panel A, and scenario 1 can produce patterns depicted by dark or light grey bars in panel A, depending on the relative strengths of the repressing and promoting pathways. Grey-scale indicates the activity of the gene, with black being active and grey being inactive, due to mutation or repression. Based on the results of this study, locus A could be interpreted as *FCA* and *FPA*, while locus B could be interpreted as *FY* or *FVE*. (C) Inferred genetic pathways for flowering (far left) and germination for scenarios 1 and 2. Dotted lines indicate relationships observed in the germination pathways that were not observed in the flowering pathways (i.e. the non-concordant components of the pathways). Here, *FLC* is included as a hypothesis only, since this study does not directly test the involvement of *FLC*. *FLC* is depicted when results do not conflict with the interpretation that autonomous-pathway genes repress *FLC* to repress flowering and enhance germination. '?' indicates a component that cannot be explained by autonomous-pathway genes repressing *FLC*; this component may be an unknown gene that acts independently of the *FLC* pathway, or it may be *FLC* that is upregulated, as opposed to repressed, by autonomous-pathway genes. Temperatures indicated in parentheses indicate inferred pathways based on findings only in that temperature.

Most of the genes studied here impeded germination when functional under at least some conditions. This basic pattern is consistent with their function as repressors of *FLC*, since *FLC* is known to promote germination (Chiang *et al.*, 2009; Blair *et al.*, 2017). *FY* has been previously shown to influence seed dormancy, and these results are consistent with that finding (Jiang *et al.*, 2012; Cyrek *et al.*, 2016). In particular, Jiang *et al.* (2012) found that the *fy-1* mutant had reduced dormancy (higher germination), but also higher levels of the dormancy-inducing hormone ABA, suggesting that *fy-1* is insensitive to ABA. However, the domains of *FY* that are essential for the control of flowering were not essential for ABA-induced dormancy, suggesting different domains of the gene are involved in the regulation of flowering versus germination. Although *FLC* expression was not shown to be altered in dry seeds in the *fy-1* mutant in their study, it cannot be ruled out that *FLC* is involved in the dormancy phenotype, because *FLC* expression increases during the late stages of seed development when dormancy is induced (Chiang *et al.*, 2009). Therefore, although *FY* regulates seed germination via the same hormone as is involved in its regulation of flowering (ABA), it is still unclear whether it regulates both traits through *FLC*. Cyrek *et al.* (2016) found evidence that *FY* may influence germination through mediating the alterative polyadenylation of transcripts of *DOG1*, a major regulator of seed dormancy (Bentsink *et al.*, 2006). Thus, *FY* may interact directly with other dormancy genes, independently of *FLC*. However, although no interactions between *DOG1* and *FLC* have been documented, interactions between

these two genes in the regulation of germination have not been investigated. Despite interactions of *FY* with other dormancy genes, it cannot be ruled out that *FLC* is also involved.

In general, disruption of autonomous-pathway genes increased germination, but the contribution of some genes varied with temperature or duration of after-ripening. This result is consistent with the temperature- and dormancy-dependent contribution of *FLC* itself to germination (Blair *et al.*, 2017). Because *FLC* influences germination in concert with other dormancy-regulating genes (Holdsworth *et al.*, 2008; Footitt *et al.*, 2011, 2014), its effect is most pronounced at intermediate levels of dormancy and is contingent on temperature. When dormancy is high and temperatures are non-permissive for germination, *FLC* alone does not overcome those inhibitory conditions to induce germination; when dormancy is very weak and germination conditions are very permissive, all seeds can germinate regardless of *FLC* abundance (Blair *et al.*, 2017). Here, consistent with those results, we saw that when wild type seeds had maximum germination (weak dormancy and permissive temperature for germination – in this experiment, after-ripened seeds at 22 °C), disruption of autonomous-pathway genes had no detectable promotive effect on germination. Moreover, it was only under these conditions that the inhibitory effect of disrupting *FPA* was detectable.

Although most genes in their active form operated to inhibit germination, some evidence for opposing effects was found. This is not expected based on the function of autonomous-pathway genes solely as repressors of *FLC*, since *FLC* promotes germination (Chiang *et al.*, 2009; Blair *et al.*, 2017). Specifically, *FCA* and *FPA* appear to have some positive contribution to germination under some circumstances. A recurrent pattern was that single *fca* or *fpa* mutants did not have altered germination, single *fy* or *fve* mutants had higher germination, but double mutants (*fca* or *fpa* combined with *fy* or *fve*) had lower germination than the *fy* or *fve* single mutants (Fig. 2A). One interpretation of this pattern is that *FY* and *FVE* repress the ability of *FCA* and *FPA* to promote germination in a linear epistatic relationship (Fig. 2, scenario 2). If functional *FCA* (or *FPA*) promotes germination, a single *fca* (or *fpa*) mutation would have no effect if *FY* and *FVE* are already repressing it. If *FY* or *FVE* is disrupted, however, that repression of *FCA* (or *FPA*) would be lost, so germination would be enhanced. In the double mutants, repression of *FCA* (or *FPA*) is lost, but so is its ability to promote germination, leading to a reduction of germination compared to the single *fve* or *fy* mutant as well as no significant difference from the wild type. A second interpretation of this pattern is that *FY* and *FVE* strongly repress germination, possibly through interacting with *FCA* (and *FPA*) as they do during flowering, but that *FCA* (and *FPA*) weakly promotes germination independently of *FY* and *FVE* (Fig. 2, scenario 1). In this scenario, a single *fca* (or *fpa*) mutant may have no discernible effect if active *FY* and *FVE* is strongly repressing germination. If those repressing genes are disrupted, germination would increase; but if the promoting pathway of *FCA* (or *FPA*) is also disrupted, in the absence of the overwhelming repression by *FY* or *FVE*, then germination would be reduced in the double mutant compared to the *fy* or *fve* single mutants. In this case, germination may not be reduced to the level of the wild type, if the pathway that promotes germination is weaker than the pathway that represses it (as seen in *fca/fve* and *fpa/fve* double mutants in some conditions). This interpretation is consistent with the finding that *FCA* and *FPA* may be involved in the siRNA-mediated silencing of targets other than *FY* (Bäurle *et al.*, 2007). Moreover, scenario 1 is more concordant with the inferred relationships in the regulation of flowering than scenario 2. In summary, the pattern suggests that *FCA* and *FPA* in some way promote germination through pathways that are repressed or overwhelmed by *FY* and *FVE*.

The observation that disruption of *FCA* and *FPA* decreases germination in some circumstances suggests that these genes do not affect germination exclusively through the repression of *FLC*. One possibility is that these genes may act in germination-promoting pathways that operate independently of *FLC* under some conditions. Alternatively, when their partners are non-functional, they may themselves promote *FLC* expression. Finally, it is possible that through complex interactions with other genes, they may repress repressors of *FLC*. Distinguishing between these possibilities would require quantification of *FLC* expression in these mutants as well as phenotypic measurements in combinatorial mutants between these autonomous-pathway genes and *FLC*, as well as higher-order autonomous-pathway mutations combined with mutation of *FLC*.

Other pleiotropic effects of autonomous-pathway genes have been documented, including effects on vigour, chlorophyll accumulation, leaf and inflorescence shape, cold-stress response, defence against fungal pathogens, and circadian clock regulation (Martinez-Zapater *et al.*, 1995; Koornneef *et al.*, 1998; Meier *et al.*, 2001; Kim *et al.*, 2004; Henderson *et al.*, 2005; Salathia *et al.*, 2006; Veley and Michaels, 2008; Lyons *et al.*, 2015). In some cases, effects of autonomous-pathway genes have been shown to occur independently of *FLC* regulation (Lyons *et al.*, 2015). For instance, in mutants without functional *FLC*, mutant *fpa* genotypes showed some effect of flowering (Michaels and Amasino, 2001), and *FPA* was shown to interact with genes not in the *FLC* flowering pathway (Koornneef *et al.*, 1998). Genetic targets other than *FLC* have been suggested based on microarray studies (Marquardt *et al.*, 2006), and *FCA* and *FPA* have been implicated in the siRNA-mediated silencing of other single- or low-copy loci (Bäurle *et al.*, 2007). The observation that the function of several autonomous-pathway genes is conserved in diverse taxa, including monocots (Lee *et al.*, 2005; Winichayakul *et al.*, 2005; Baek *et al.*, 2008; Abou-Elwafa *et al.*, 2011; Sun *et al.*, 2012; Hu *et al.*, 2014), even when *FLC* is not conserved, further suggests that these genes may act independently of *FLC* (Simpson, 2004). Therefore, autonomous-pathway genes may operate independently of *FLC*, but the extent to which pleiotropic effects of autonomous-pathway genes are independent of *FLC* remains unknown.

Autonomous-pathway genes sometimes interacted non-additively to influence germination. In general, these interactions are similar to those observed for flowering-time regulation (Fig. 2C, especially scenario 1). Specifically, *FCA* and *FPA* interacted with *FVE* and *FY* non-additively, and *FY* and *FVE* interacted with each other to influence germination. The overall similarity of the direct contributions and interactions of these genes in their regulation of flowering and germination is notable, and suggests conservation of function in their regulation of flowering and germination. However, the observation that *FCA* and *FPA* can conditionally promote germination suggests some divergence of function between the regulation of flowering and germination by the autonomous pathway, since

such antagonistic effects of these genes have not been documented in their regulation of flowering. This experiment did document some evidence that *FPA* may delay flowering in the absence of functional *FVE*, suggesting possible concordance across flowering and germination even of this antagonistic function. Note that such conservation of function across development is not always found in genes that pleiotropically regulate flowering and germination. For example, some genes in the vernalization pathway were found to be functionally conserved across flowering and germination (specifically *FRI* and *VIN3*) but others were not (*VIP3* and *VRN2*; Auge *et al.*, 2017). Thus, even though genes share the regulation of different life stages, they may do so in different ways or through different pathways.

Regarding autonomous-pathway genes, not only are individual genes pleiotropic across germination and flowering pathways, but the overall structure of the pathway appears to be highly, if not completely, conserved across development (Fig. 2C, scenario 1). Such concordance of genetic pathways in the regulation of these two traits suggests that genes in the autonomous pathway strengthen genetic correlations between these traits. Specifically, they promote flowering, but impede germination. In winter annuals such as *A. thaliana*, this could be manifest as the induction of flowering in spring and the delay of germination until autumn – the typical phenology expressed by winter annuals. However, if flowering is induced in autumn, germination could be postponed until spring, which is not likely to be adaptive (Donohue *et al.*, 2005). Thus, the adaptive value of this pleiotropy probably depends on whether other genes in the flowering pathway permit autumn flowering or enforce the winter-annual habit of flowering in the spring.

In conclusion, evidence of pleiotropy of genes in the autonomous flowering-time pathway was found, such that both flowering and germination appear to be regulated by these genes. They do so primarily in a manner consistent with their function as repressors of *FLC* expression, although some genes exhibited other modes of function. Moreover, the interactions among those genes were found to be largely concordant between flowering and germination regulation. Thus, a functional conservation is apparent across development for these genes, which can contribute to correlations among flowering and germination time and influence overall life cycles.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: ARBC stock numbers for mutants used in this study. Table S2: Effects of mutation on flowering time. Table S3: Effects of mutation on germination proportions. Figure S1: Differences between mutant genotypes and the L*er* wild type in bolting time. Figure S2: Differences among genotypes in flowering time and germination for combinatorial pairs of mutations.

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