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Quantitative Modulation of Polycomb Silencing Underlies Natural Variation in Vernalization

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***Arabidopsis thaliana* accessions have adapted to growth in a wide range of climates. Variation in flowering and alignment of vernalization response with winter length are central to this adaptation. Vernalization involves the epigenetic silencing of the floral repressor *FLC* via a conserved Polycomb (PRC2) mechanism involving trimethylation of Lys²⁷ on histone H3 (H3K27me3). We found that variation for response to winter length maps to cis polymorphism within *FLC*. A rare combination of four polymorphisms localized around the nucleation region of a PHD-Polycomb complex determines a need for longer cold. Chromatin immunoprecipitation experiments indicate that these polymorphisms influence the accumulation of H3K27me3 in *Arabidopsis* accession Lov-1, both at the nucleation site and over the gene body. Quantitative modulation of chromatin silencing through cis variation may be a general mechanism contributing to evolutionary change.**

The molecular basis of natural variation has been defined for relatively few traits. We have focused on natural variation in vernalization, the acceleration of flowering by prolonged cold that aligns flower and seed development with favorable environmental conditions. Vernalization is an inherently quantitative process, with increasing periods of cold giving progressive acceleration of flowering. In *A. thaliana* this is the result of cell-autonomous stochastic switching of the *FLC* locus to an epigenetically silenced state, with longer cold increasing the probability of that switch occurring (1). Extensive natural variation exists in *A. thaliana* accessions for how much cold is required to give complete epigenetic silencing of *FLC* (2, 3). In some accessions, a vernalization period of 4 to 6 weeks of cold gives incomplete *FLC* silencing; the gene then reactivates after transfer back to warm conditions, and flowering is delayed. This variation has been mapped to several major loci including *FLC* itself (3, 4).

To better understand the molecular basis of this natural variation, we focused on two vernalization-requiring *A. thaliana* genotypes: Lov-1, an accession collected from the northern range of the species in northern Sweden (62.5°N) (5), and *FRI* Col, the reference Columbia accession containing an introgressed active *FRIGIDA* allele (6). Lov-1 requires 9 weeks of cold to fully saturate the vernalization requirement, thereby epigenetically silencing *FLC* and enabling activation of the *A. thaliana* floral promoter *FT* (Fig. 1A). Partial vernalization of Lov-1 (4 weeks of cold) led to incomplete epigenetic silencing and reactivation of the Lov-1 *FLC* allele upon transfer of plants back to warm conditions, no activation of *FT*, and thus late flowering. In contrast, 4 weeks of cold was sufficient to fully saturate the vernalization requirement in *FRI* Col, enabling *FT* activation and early flowering in favorable conditions (Fig. 1A).

Variation in the ability of a fixed length of cold to induce epigenetically stable *FLC* silencing is therefore a major determinant of flowering time variation in these two genotypes. A substantial fraction of this vari-

ation mapped to a large genomic interval including *FLC* (3, 4). We continued to fine-map the quantitative trait locus to an interval of 0.5 Mb containing 182 genes (fig. S1). After experiencing 4 weeks of cold, the flowering of lines carrying the Lov-1 *FLC* allele introgressed six times into the *FRI* Col background was significantly delayed (39.5 ± 1.4 days versus 29.9 ± 1.8 days for *FRI* Col; $n = 10$). We undertook complementation experiments transforming Col and Lov-1 *FLC* ~10-kb genomic clones into the common background *FRI flc-2* and found that transformants carrying the Lov-1 *FLC* allele flowered significantly later after 4 weeks of cold than did those carrying the Col *FLC* allele (Fig. 1B). In addition, the Lov-1 *FLC* expression was reactivated relative to the stable silencing of the Col *FLC* allele in a Col background (Fig. 1C). Together, these data suggest that cis variation at the Lov-1 *FLC* allele modulates the length of cold required to achieve the epigenetically stable silencing of *FLC*, necessary for up-regulation of expression of the floral promoter *FT*.

The Lov-1 and Col *FLC* genomic clones used in the complementation experiments differ by 23 single-nucleotide polymorphisms (SNPs) and 16 indels, all in noncoding regions (table S1 and Fig. 2). We generated different combinations of Lov-1 and Col *FLC* alleles to map the region(s) responsible for the variation in silencing. Ten such mix-and-match alleles were transformed into *FRI flc-2* plants (Fig. 2). To reduce the effect of between-transformant variability, we pooled a large number of seedlings from multiple transformants (between 23 and 49 independent lines) to obtain quantitative *FLC* expression data before the cold, at the end of the cold, and after transfer back to warm conditions (Fig. 2 and figs. S2 and S3). The minimal regions of the Lov-1 *FLC* allele sufficient to confer reactivation after 4 weeks of cold were regions b and c, and both together showed a stronger reactivation (Fig. 2, constructs 3, 8, and 9). Only four polymorphisms localize to these regions—two just upstream of the transcription start, the other two at the beginning of intron 1. These two regions are included in the Polycomb silencing nucleation region, where the PHD-PRC2 (core PRC2 plus three PHD proteins) associates during the cold (7) and where H3K27me3 quantitatively accumulates (1). Inclusion of other Lov-1 sequences, especially the promoter region (region a), enhanced the degree of reactivation (Fig. 2, compare constructs 3 and 6), but the polymorphisms in the promoter were not sufficient by themselves to modulate *FLC* silencing after cold (Fig. 2, construct 5). The lack of reactivation in construct 4 suggests that other combinations of polymorphisms can also influence the silencing (Fig. 2).

We then looked at the frequency and distribution of the four polymorphisms from regions b and c in a selected set of worldwide annual *A. thaliana* accessions requiring different lengths of vernalization (2) (table S2). At positions -121 and +326 relative to the transcription start site, the Lov-1 SNP occurs with high frequency in the populations analyzed. The Lov-1 single-base pair indel at position -56 occurs with medium frequency, whereas the Lov-1 SNP at +598 is rare. The latter is present in only one other accession, Lov-5, which had been found very close to

the Lov-1 collection site. Only two of the polymorphisms are significantly associated with vernalization response (−121 and +598; $P < 0.01$, Mann-Whitney test; table S3). However, the −121 Lov-1 SNP (the more common type) also associates with flowering time in nonvernalizing conditions. These results suggest that the SNPs can independently influence flowering time in both nonvernalized and vernalized plants, and that it is the combination of specific polymorphisms that leads to the characteristic vernalization response of Lov-1.

Analysis of the phases of vernalization in *A. thaliana* has shown that the core PRC2 is associated with *FLC* at all times: before cold, during cold, and after stable silencing has occurred upon return to warm conditions (7–9). The switching of epigenetic states is initiated by cold-induced formation of a PHD-PRC2 complex and by a localized increase in H3K27me3 in the nucleation region. Upon transfer to warm temperature, a subset of the PHD proteins associates with PRC2 over the whole locus, concurrent with a substantial increase in H3K27me3 over the same region. To understand how the polymorphisms that map near this nucleation region might affect the epigenetic silencing, we analyzed H3K27me3 accumulation over Lov-1 *FLC* at different stages of vernalization (Fig. 3). H3K27me3 levels were found to be much lower over the whole Lov-1 *FLC* locus before vernalization relative to *FRI* Col (Fig. 3A), and it took 9 weeks to reach equivalent H3K27me3 levels in the nucleation region in Lov-1 versus 4 weeks in *FRI* Col. This led to correspondingly lower levels of H3K27me3 over the remainder of the gene after transfer to warm conditions in Lov-1 *FLC* (Fig. 3B). We then checked whether the lower initial levels of H3K27me3 were also found in *FRI* Col lines carrying the Lov-1 *FLC* allele introduced via either introgression or transformation. In both situations, H3K27me3 level was reduced before and after vernalization (Fig. 4), showing that the phenotype was a consequence of cis variation in Lov-1.

How the sequence polymorphisms influence the multiple aspects of H3K27me3 dynamics awaits a fuller understanding of the nucleation process. The four SNPs are not in sequences with homology to known Polycomb response elements or in the region encoding the sense noncoding RNA COLDAIR (10). However, the −121 and −56 SNPs do overlap with the promoter of the sense transcript and the last exon of a spliced variant of COOLAIR, the *FLC* antisense transcript up-regulated by short cold (11) (fig. S4). We looked at *FLC* antisense expression, and although we could detect no difference before vernalization, we observed a higher increase in the distally polyadenylated antisense transcript in Lov-1 relative to that in *FRI* Col after 2 weeks of cold (fig. S5). It is intriguing to speculate that this form of the antisense transcript might antagonize Polycomb complex recruitment (12). This could be due to secondary structure changes caused by the −121 SNP, as suggested by RNAfold predictions (13). The −121, −56, and +326 SNPs are within a region previously shown to be required for repression by vernalization (14). The +598 SNP is within a different functional domain required for maintenance of the silencing (14) and is flanked by two putative binding sites of B3 transcriptional regulators (15). Thus, it is likely that the SNPs influence multiple aspects of the nucleation mechanism.

Cis variation at enhancers or at transcription factor binding sites has played an important role in morphological evolution (16). Our results indicate that cis polymorphism leads to quantitative modulation of a Polycomb silencing mechanism and that this variation alters developmental timing in *A. thaliana* accessions. The diversity of targets regulated by the Polycomb system presumably constrains changes in the trans factors (17, 18). Multiple polymorphisms appear to contribute to the overall effect, with some occurring widely and others infrequently in the population, and the combination of these polymorphisms may be important for the evolution of this trait (19). More generally, our work suggests that quantitative modulation of chromatin silencing mechanisms through cis polymorphisms could be a general mechanism under-

lying adaptation of many organisms to changing environments. Evolution of flowering time and vernalization response is an important component of adaptation in *A. thaliana* (20), and the extent of variation of this trait may well explain the much broader geographical distribution of this species compared to its relatives (21).

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Supplementary Materials

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Materials and Methods

Figs. S1 to S5

Tables S1 to S3

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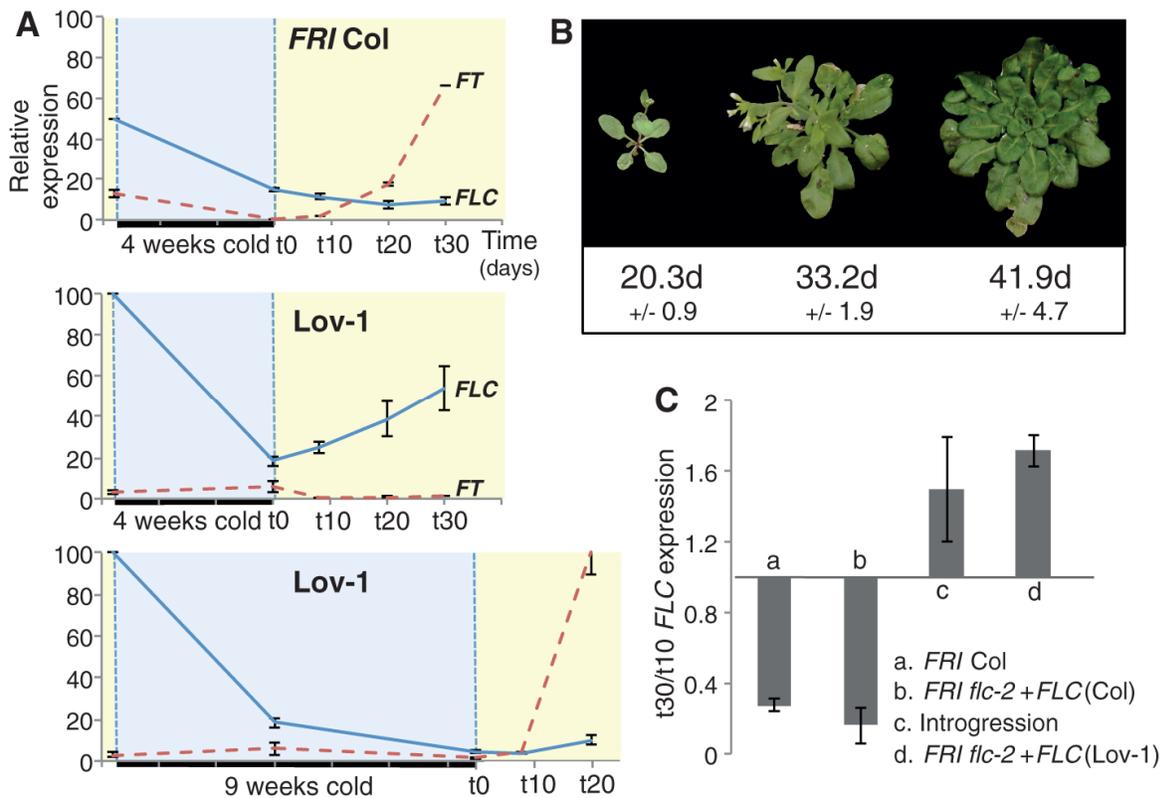


Fig. 1. *FLC* cis variation delays the vernalization response. **(A)** *FLC* and *FT* expression (solid blue line and red dashed line, respectively) before and after a vernalization treatment of 4 weeks (top and middle) or 9 weeks (bottom) followed by 0, 10, 20, and 30 days of warm (t0, t10, t20, and t30). **(B)** Top: Photograph of *FRI flc-2*, *FRI flc-2 + FLC(Col)*, and *FRI flc-2 + FLC(Lov-1)* plants (left to right) vernalized for 4 weeks and then grown in warm long-day conditions for 12 days (*FRI flc-2*) or 30 days [*+FLC(Col)*, *+FLC(Lov-1)*]. Bottom: Average flowering time (d, days to flower) of 10 individuals for each corresponding genotype. **(C)** *FLC* expression shown as a ratio of t30/t10 values after 4 weeks of vernalization treatment. A value greater than 1 denotes an increase in *FLC* expression after return to warm conditions. Introgression is the *FLC(Lov-1)* genomic region described in fig. S1 in *FRI Col*. Error bars are SEM from three biological replicates.

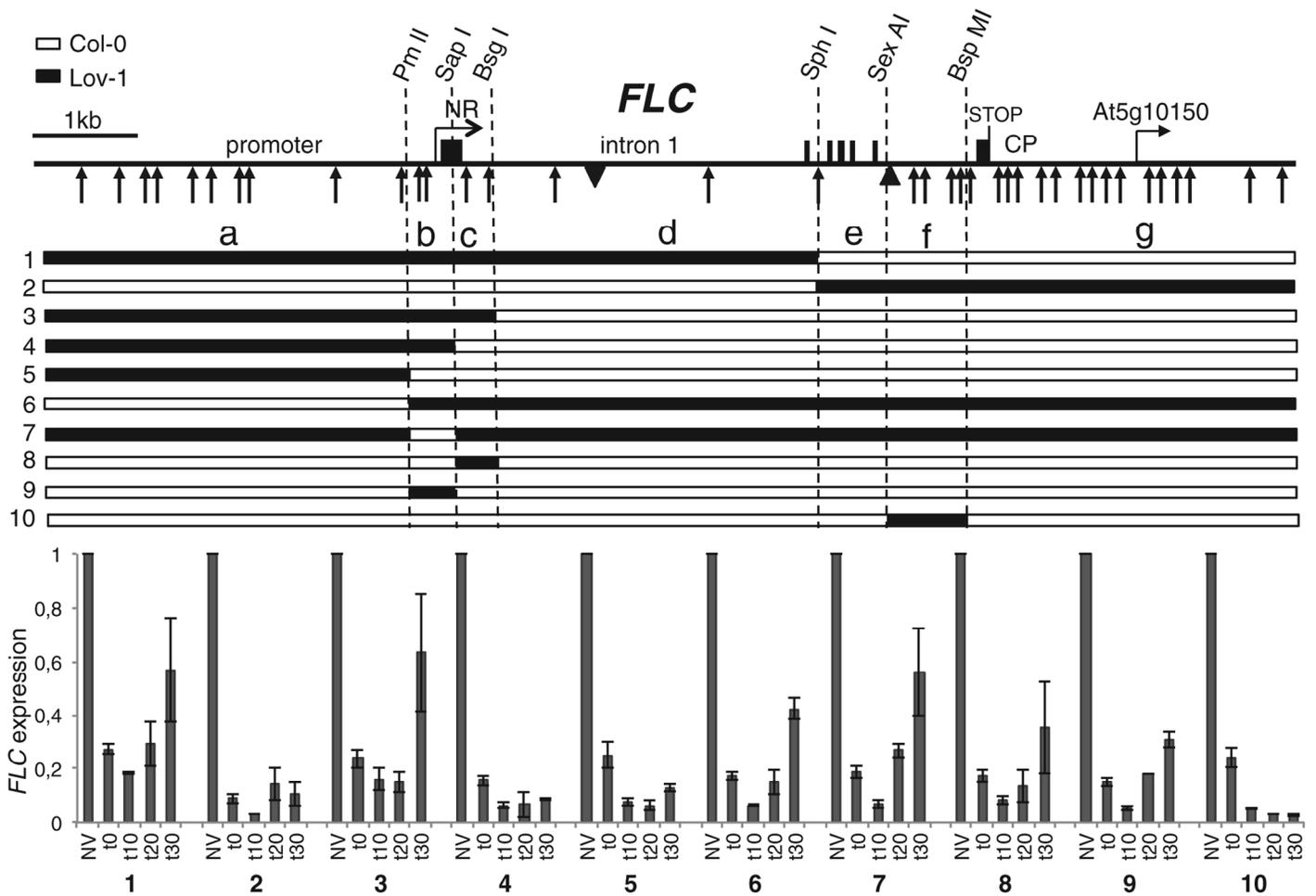


Fig. 2. Mix-and-match *FLC* alleles define polymorphisms contributing to variation in vernalization response. Top: *FLC* sequence schematic showing exons (black boxes), positions of SNPs and short indels (arrows), insertion (triangle), deletion (inverted triangle), and restriction sites used for the mix-and-match constructs (with restriction enzymes named). Rows 1 to 10 show composition of the mix-and-match constructs (white, Col sequence; black, Lov-1 sequence). Bottom: *FLC* expression analysis before (NV) and after a treatment of 6 weeks of vernalization followed by 0, 10, 20, and 30 days of warm (t0, t10, t20, and t30) in the 10 different mix-and-match lines. A pool of 23 to 49 independent T2 transformant lines was used for each construct. Error bars are SEM from three biological replicates.

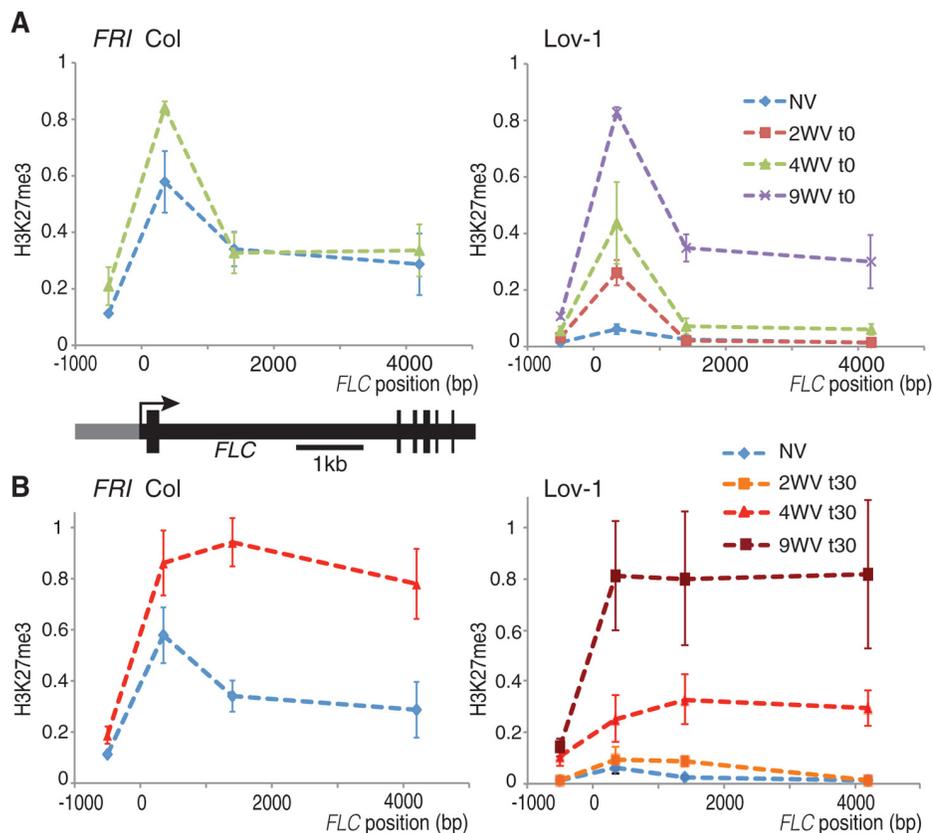


Fig. 3. H3K27me3 accumulation at *FLC* is delayed in *Lov-1*. **(A)** H3K27me3 profile across *FLC* for *Col* (left) and *Lov-1* (right) plants before vernalization (NV) and after 2, 4, or 9 weeks of cold. **(B)** H3K27me3 profile across *FLC* for *Col* (left) and *Lov-1* (right) plants before vernalization (NV) and after 2, 4, or 9 weeks of cold plus 30 days (t30) of growth in warm long-day conditions. Error bars are SEM from three biological replicates.

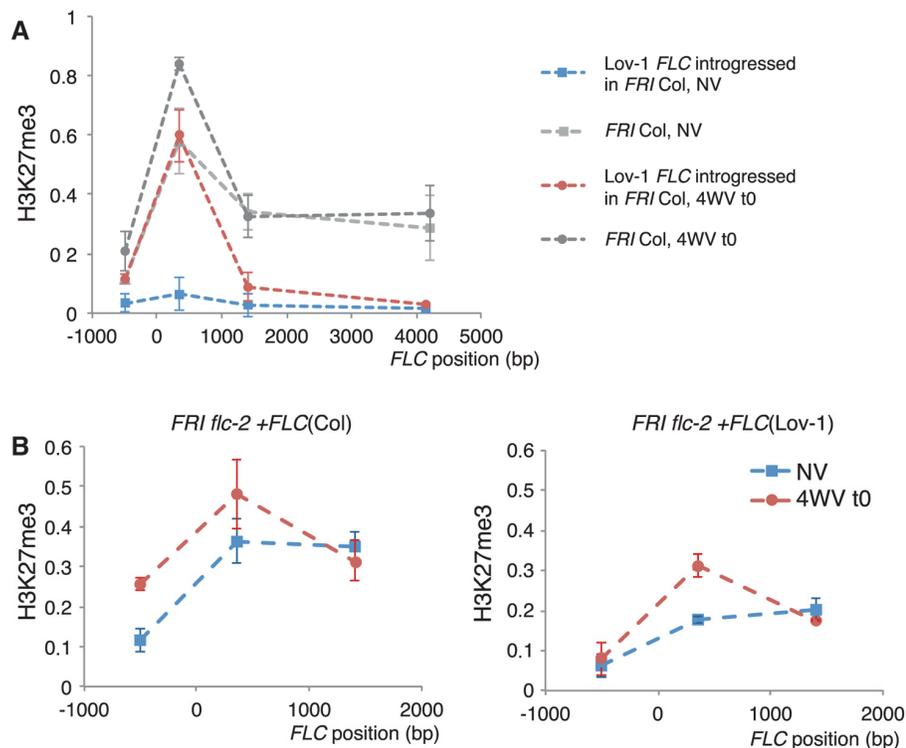


Fig. 4. Cis variation at *FLC* results in lower H3K27me3 levels. **(A)** H3K27me3 profile across *FLC* for nonvernalized (NV) plants and after 4 weeks of cold (4WV t0) in *FRI Col* plants or *Lov-1 FLC* introgressions in *FRI Col* background. **(B)** H3K27me3 profile at *FLC* in regions b and c for NV and 4WV t0 plants in *FRI flc-2 + FLC(Col)* pooled T2 transgenics (left) and *FRI flc-2 + FLC(Lov-1)* pooled T2 transgenics (right). Error bars are SEM from three biological replicates.