Antisense-mediated *FLC* transcriptional repression requires the P-TEFb transcription elongation factor

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The functional significance of noncoding transcripts is currently a major question in biology. We have been studying the function of a set of antisense transcripts called *COOLAIR* that encompass the whole transcription unit of the *Arabidopsis* floral repressor *FLOWERING LOCUS C (FLC)*. Alternative polyadenylation of *COOLAIR* transcripts correlates with different *FLC* sense expression states. Suppressor mutagenesis aimed at understanding the importance of this sense–antisense transcriptional circuitry has identified a role for *Arabidopsis* cyclin-dependent kinase C (CDKC;2) in *FLC* repression. CDKC;2 functions in an *Arabidopsis* positive transcription elongation factor b (P-TEFb) complex and influences global RNA polymerase II (Pol II) Ser^2 phosphorylation levels. CDKC;2 activity directly promotes *COOLAIR* transcription but does not affect an *FLC* transgene missing the *COOLAIR* promoter. In the endogenous gene context, however, the reduction of *COOLAIR* transcription by *cdkc;2* disrupts a *COOLAIR*-mediated repression mechanism that increases *FLC* expression. This disruption then feeds back to indirectly increase *COOLAIR* expression. This tight interconnection between sense and antisense transcription, together with differential promoter sensitivity to P-TEFb, is central to quantitative regulation of this important floral repressor gene.

lncRNA | autonomous pathway | transcriptional regulation | chromatin silencing

We are investigating the role of specific antisense transcripts in gene regulation through our analysis of the regulation of expression of *Arabidopsis thaliana FLOWERING LOCUS C* (*FLC*), an important regulator of flowering time (1, 2). Multiple genetic pathways have been defined that regulate *FLC* expression; some function in parallel whereas others function antagonistically. FRIGIDA (FRI) up-regulates *FLC* expression, causing plants to overwinter vegetatively (3). FRI function is antagonized by vernalization, a process through which prolonged cold epigenetically silences *FLC* (4–7). Acting in parallel with vernalization to repress *FLC* expression is the autonomous pathway. The autonomous pathway was initially characterized through mutations specifically affecting flowering time but has subsequently been shown to regulate many other targets in the *A. thaliana* genome (8–10). The autonomous pathway is composed of a number of factors: RNA-binding proteins FCA (11), FPA (12), and FLK (13, 14), RNA 3′ processing factors FY (15–17), CstF64, and CstF77 (18), a histone 3 lysine 4 (H3K4) demethylase FLD (19, 20), a homolog of MSI1 (multicopy suppressor of *ira*1) FVE (21), and a homeodomain protein LUMINIDEPENDENS (LD) (22). These factors link alternative processing of a set of antisense transcripts produced at the *FLC* locus, collectively termed *COOLAIR*, with chromatin modifications in the *FLC* gene body (8). FCA, FY, and FPA promote proximal polyadenylation of *COOLAIR* transcripts (18), FLD-dependent H3K4me2 demethylation across the body of the gene, and low *FLC* transcription (23). Their loss results in distal polyadenylation in *COOLAIR*, increased H3K4 methylation across the gene, and high expression (24), but how *COOLAIR* processing is linked to *FLC* transcription is still not fully understood. *COOLAIR* is also regulated transcriptionally via an extensive R-loop that covers the *COOLAIR* promoter and first exon (25).

To gain a better understanding of the sense–antisense mechanism regulating *FLC*, we have undertaken suppressor mutagenesis and have identified a requirement for cyclin-dependent kinase C (CDKC;2). This protein is an *Arabidopsis* ortholog of a component of the positive transcription elongation factor b (P-TEFb) (26–29). P-TEFb regulates transcription elongation and integrates mRNA synthesis with histone modification, pre-mRNA processing, and mRNA export (30). *Arabidopsis* CDKC;2 has previously been shown to be important for flowering time control and plant virus infection (26) and to colocalize with spliceosome components in nuclear bodies (31). Here, we show that CDKC;2 functions globally in the *A. thaliana* genome to influence the phosphorylation status of RNA polymerase II (Pol II), as anticipated from a P-TEFb function. We also investigate CDKC;2 function on sense *FLC* and *COOLAIR* transcription through analysis of transgenic lines, where both are expressed independently of each other. This analysis established that *cdkc;2* specifically reduces transcription of *COOLAIR*, which indirectly up-regulates *FLC* expression through disruption of a *COOLAIR*-mediated repression mechanism. The feedback mechanisms that link sense and antisense transcription in the endogenous gene context (18) then indirectly increase *COOLAIR* expression. This sensitivity of the *COOLAIR* promoter to P-TEFb function suggests that the antisense transcription is the driver quantitatively regulating expression levels at *FLC*.

Significance

Noncoding transcripts are found in high complexity but low abundance in most genomes, but their functional relevance is unclear. We investigate the function of a set of antisense transcripts expressed from an important floral repressor gene in *Arabidopsis*. Different polyadenylated forms of the antisense transcripts correlate with high or low expression states of the floral repressor gene. We now identify a mutation in a conserved transcription elongation factor that specifically disrupts the antisense transcription. The direct reduction of antisense transcription releases a repression mechanism that indirectly increases expression of both the floral repressor gene and antisense expression. This study reveals tight interplay between sense and antisense transcription and a mechanism that could have a widespread role in quantitative gene regulation.

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Results

***sof77*, a Mutation in *CDKC;2*, Increases *FLC* Expression.** We used suppressor mutagenesis to identify components mediating repression of *FLC* expression. The Landsberg *erecta* (L*er*) progenitor line expresses high levels of *FCA* in combination with *FRI* and an *FLC (Col)::LUC* transgene (18, 23). This line provides a system where the function of pathways both promoting and repressing *FLC* expression is enhanced relative to rapid-cycling genotypes normally used in *Arabidopsis* mutagenesis, so giving a sensitized state where even subtle effect mutations can easily be detected. A mutant called *sof77* was identified that increased FLC::LUC expression and delayed flowering (Fig. 1 *A*, *B*, *D*, and *E*). Genetic mapping and sequencing revealed that *sof77* carried a mutation that changes the ACA threonine codon (amino acid 198) into ATA encoding isoleucine in *CDKC;2* (AT5G64960) (Fig. 1*C*). We named the mutation *cdkc;2–3* because the two transferred DNA (T-DNA) insertion alleles in Col were named *cdkc;2–1* and *cdkc;2–2* (Fig. S1*A*) (26). CDKC;2 (AT5G64960) is one of two *Arabidopsis* orthologs of the CDK9 component of P-TEFb, the positive transcription elongation factor b, the other being CDKC;1 (AT5G10270) (26, 27, 29).

We analyzed the involvement of CDKC;2 in *FLC* repression in a genotype not sensitized to FCA action by out-crossing the *35S::FCAγ* and *FRI* transgenes. *cdkc;2–3* elevated endogenous *FLC* mRNA levels by ~2.5 fold and flowered later with approximately twice as many leaves compared with the L*er* wild type (Fig. 1 *D* and *E*). This phenotype is much weaker than *fca* but demonstrates that CDKC;2 is involved in repressing *FLC* expression in wild-type *Arabidopsis* plants. Expression of HA- and FLAG-tagged CDKC;2 under its native regulatory sequences complemented the late flowering of the *cdkc;2–3* mutant (Fig. 1 *D* and *E* and Fig. S1*C*). Allelic analysis using the T-DNA insertion mutant (SALK_029546, *cdkc;2–2*) also supported the enhanced FLC::LUC signal and later flowering time being caused by loss-of-function of the *CDKC;2* gene.

As well as late flowering, *cdkc;2–3* was associated with changed color of the leaves, slow growth, curved siliques, and aborted seeds, suggesting that CDKC;2 function is generally important for plant growth and development (Fig. S1 *C–F*). These mutant phenotypes reveal that CDKC;1 cannot cover loss of CDKC;2 function, perhaps because in silico gene expression analysis suggests that CDKC;1 expression could be much lower throughout development (Fig. S2).

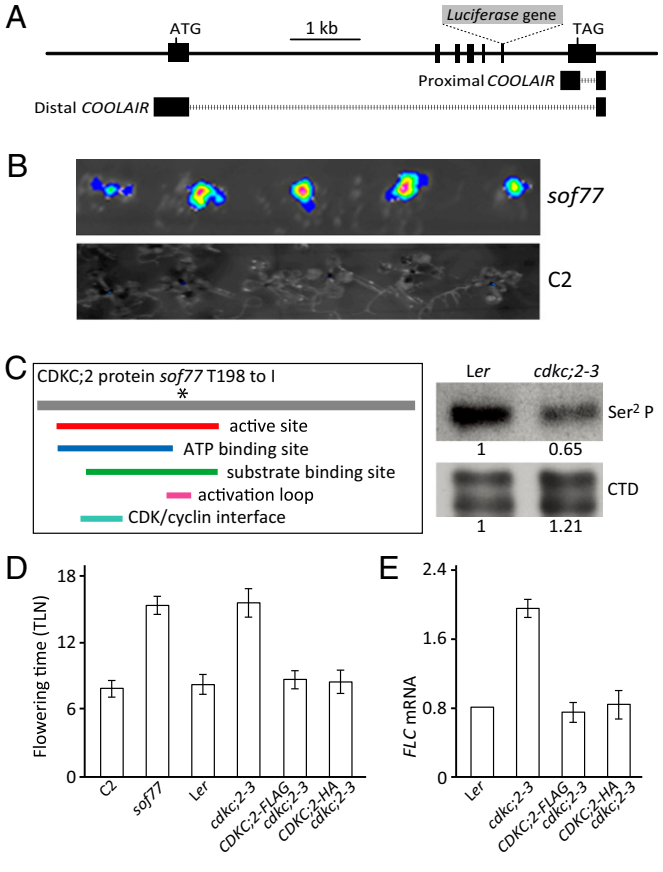

Fig. 1. Characterization of *cdkc;2*. (*A*) Schematic illustration of the *FLC::LUC* construct. (*B*) *sof77* shows high FLC::LUC in young seedlings compared with the C2 progenitor containing *FRI*, *35S::FCAγ*, *FLC::LUC*. (*C*) Domains within CDKC;2. The * shows the position of the mutation in *cdkc;2–3* plus Western blots of protein extracted from seedlings of *cdkc;2–3* and L*er*. Membranes were probed with antibodies against CTD Ser^2 P Pol II (3E10) or total Pol II (8WG16). (*D* and *E*) The CDKC;2 HA or FLAG-tagged genomic fusions complemented the *cdkc;2–3* mutant phenotype. Flowering time values in *D* are means ± SD (n = 20); *FLC* expression values in *E* are means ± SEM from three biological repeats.

CDKC;2 Functions in the FCA-Dependent Genetic Pathway to Down-Regulate *FLC*. We first determined whether the *cdkc;2* mutation affected expression of *FLC* regulators. We found no change in *FCA*, *FY*, *FLD* RNA expression and the feedback regulation of FCA on the processing of its own transcript, showing that the increase of *FLC* expression was not due to an effect on FCA function (Fig. S3). We undertook an epistasis analysis to test whether CDKC;2 functions in the same pathway as known autonomous pathway components, namely FCA, FY, CstF64, and FLD. We used both the *cdkc;2–3* allele in L*er* and the T-DNA insertion *cdkc;2–2* allele in Col for the analysis. *cdkc;2* was not additive with *fca*, *fld*, *fy*, or *cstf64-1* mutant alleles but was additive with active *FRI* on the basis of analysis of both *FLC* expression levels and flowering time (Fig. 2). Thus, our analysis demonstrates that CDKC;2 functions in the same genetic pathway as FCA to limit *FLC* expression.

Our previous study had shown that that autonomous pathway mutants, including *fld*, *cstf64*, and *cstf77*, increase *FLC* transcriptionally (18, 23). Using similar assays, we found that *cdkc;2–3* also caused increased *FLC* transcription. Unspliced transcript levels were higher (Fig. 3*B*), and H3K4me2 increased in the central region of the gene (regions F and G, Fig. 3*C*). H3Ac levels were higher in *cdkc;2* near the sense and antisense promoter regions (regions B, C, H, and U, Fig. 3*D*). These data would suggest that loss of CDKC;2 perturbs aspects of the same repression mechanism as other autonomous pathway mutations.

The CDKC;2–CYCT Complex Forms an *Arabidopsis* P-TEFb Complex. CDKC;2 is homologous to the CDK9 kinase subunit of human P-TEFb. P-TEFb phosphorylates the C-terminal domain (CTD) of Pol II at the Ser^2 site within the heptad repeats (26, 29). This Ser^2 posttranslational modification orchestrates the interplay between transcriptional elongation and processing of mRNA (32–35). This amino acid change in *cdkc;2–3* is within the activation loop, known to be important for substrate binding, but not involved in interaction with the cyclin component of P-TEFb (Fig. 1*C*) (36). The specific kinase activity of CDKC;2 had not been previously explored so we assayed the phosphorylation status of Pol II CTD in wild-type and *cdkc;2–3* plants. Western blot analysis using antibodies to CTD Ser^2 phosphorylated Pol II (37) and hypophosphorylated CTD showed specific reduction in levels of CTD Ser^2 phosphorylated Pol II in *cdkc;2–3* (Fig. 1*C*). This result supports a functional role for CDKC;2 as part of an *Arabidopsis* P-TEFb complex.

Yeast two-hybrid and coimmunoprecipitation analyses have suggested that the gene products from the cyclin T genes CYCT1;3, CYCT1;4, and CYCT1;5 could be the cyclin partner of CDKC;2 (26). To definitively define the cyclin partner associated with

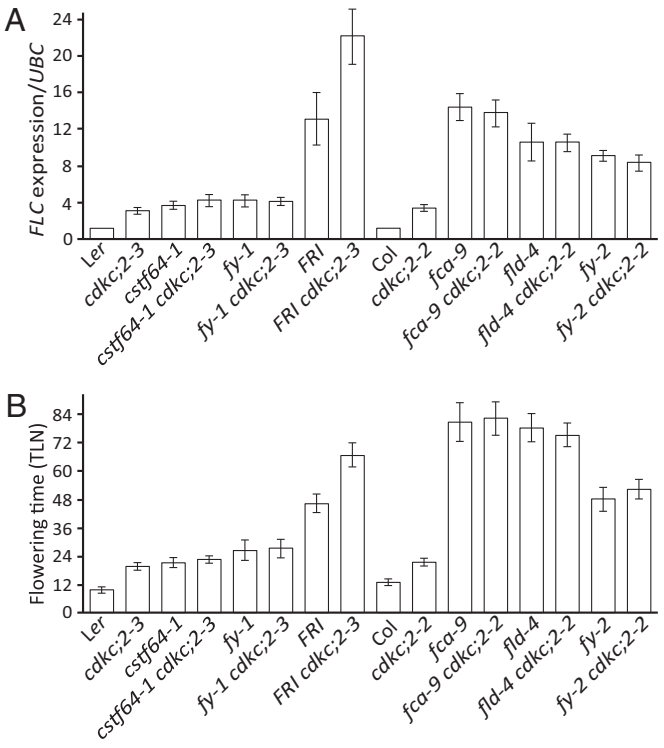

Fig. 2. CDKC;2 functions together with autonomous pathway components to repress *FLC* expression and accelerate flowering. (*A* and *B*) *cdkc;2* is not additive with *cstf64*, *fy*, *fca*, or *fld* in *FLC* repression. *FLC* expression values are means ± SEM from three biological repeats. Flowering time [total leaf number (TLN)] data are means ± SD (n = 20).

CDKC;2 in P-TEFb in planta, we immunoprecipitated a complementing CDKC;2-FLAG stably expressed in *cdkc;2–3* (Fig. 1 *D* and *E* and Fig. S1*B*) and identified associated proteins by mass spectrometry (MS). This experiment identified several peptides corresponding to CYCT1;4 and CYCT1;5, with most corresponding to CYCT1;5 (Table 1). These data suggest that the predominant partner in vivo is CYCT1;5 but that both can function in *Arabidopsis* P-TEFb activity in vivo. Consistent with a role in *FLC* regulation, a genotype deficient in CYCT1;5 and with reduced CYCT1;4 (*cyct1;5/CYCT1;4RNAi*) has been found to flower late with enhanced levels of *FLC* mRNA (26). We also detected phosphorylation of a conserved threonine in CDKC;2 by MS (Figs. S4*A* and S5). Phosphorylation of this threonine, T186, in mammals corresponding to T198 in *Arabidopsis* CDKC;2, on the activation loop in CDK9 is essential for kinase activity (36).

CDKC;2 Regulates *FLC* Expression via an Influence on *COOLAIR* Production. The requirement of CDKC;2 to repress expression of the *FLC* gene runs counter to the role of P-TEFb as a positive transcriptional elongation factor. We therefore used chromatin immunoprecipitation experiments using the lines carrying the complementing CDKC;2-HA fusion to test that CDKC;2 function at the *FLC* locus was direct (Fig. 1 *D* and *E* and Fig. S1*B*). There was a low level of enrichment across the whole locus, with the highest at the 3′ region of *FLC* near the *COOLAIR* promoter (Fig. 4*A*). The increase in *COOLAIR* expression in *cdkc;2* was associated with changed *COOLAIR* processing; relative to total levels of *COOLAIR*, proximal polyadenylation was reduced and distal polyadenylation increased (Fig. 4*C*). This alteration parallels the situation in other mutants where *FLC* expression increased (18).

Our previous work had demonstrated a role of *COOLAIR* in *FLC* regulation (38). To investigate whether the effect of CDKC;2 on *FLC* expression was mediated via *COOLAIR*, we generated an *Arabidopsis* line expressing an *FLC* transgene, where the native *FLC* 3′ region from the translation stop codon to past the poly (A) site (which corresponds to the *COOLAIR* promoter, the *COOLAIR* first intron, and the beginning of the *COOLAIR* second exon) was replaced by the 3′ untranslated region from the translation stop codon to past the poly (A) site of the *Arabidopsis rbcs3B* gene (Fig. 4*B*). The transgene, called *FLC-TEX*, complemented the *flc-2* mutation; the plants flowered late and produced even higher levels of *FLC* unspliced transcript than the Col wild type (Fig. S6*A*) (39). *FLC-TEX* produced low levels of proximally polyadenylated *COOLAIR* approximately the same as the *flc-2* parent (39) [the representative *FLC-TEX* line (no. *577*) used in this analysis differed from that used previously (39) and is shown in Fig. S6*C*] and approximately half as much total antisense transcripts as wild-type plants (Fig. S6*B*), likely to result from Pol II firing from the efficient *rbcs3B* terminator (40). The *FLC-TEX* line was crossed to a *cdkc;2* mutation (using the Col *cdkc;2–2* allele to avoid mixing genetic backgrounds, selecting for *fri* individuals, but maintaining the endogenous Columbia *FLC* allele). We found that the transgene *FLC-TEX* expression in *FLC-TEX cdkc;2* was unaffected by *cdkc;2–2*, indicating that CDKC;2 repression of *FLC* expression depends on *COOLAIR* (Fig. 4*D*). This result was obtained in a background where the expression of the endogenous *FLC* locus expressing native *COOLAIR* increased (Fig. 4*D*).

We used a *COOLAIR::LUC* fusion (25, 38), where the *COOLAIR* promoter, first exon, and first intron had been fused with an internal ribosome entry site to the firefly luciferase-coding region (*LUC*) to study the affect of *cdkc;2*, in the absence of *FLC* sense expression. This *COOLAIR::LUC* transgene mimics the behavior of the endogenous *COOLAIR* transcripts (25, 38). When combined with *cdkc;2–2*, the COOLAIR::LUC expression decreased significantly, and corresponded to reduced levels of *LUC* RNA transcript (Fig. 5*A*). Levels of CTD Ser^2 phosphorylated Pol II were significantly reduced across this *COOLAIR::LUC* transgene in the *cdkc;2–2* mutant (Fig. 5*C* and Fig. S7*A*). This result is consistent with CDKC;2 association near

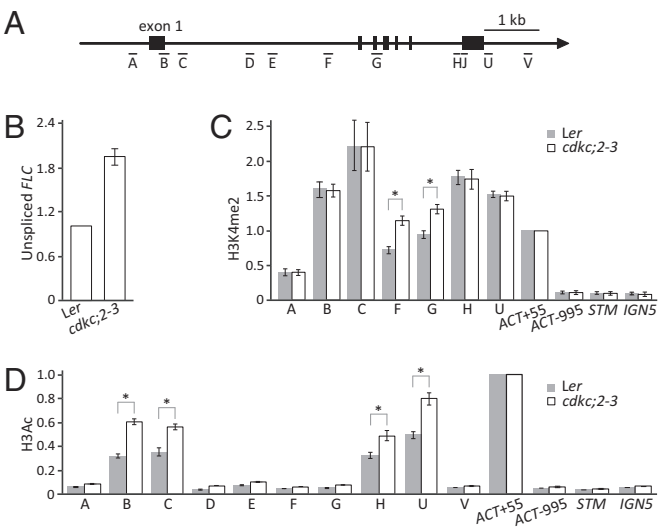

Fig. 3. *cdkc;2* increases *FLC* transcription. (*A*) Schematic of *FLC*. Vertical bars show the exons. A, B, C, D, E, F, G, H, U, and V show regions analyzed by qPCR in ChIP. (*B*) Unspliced *FLC* transcript analysis by qRT-PCR. (*C*) H3K4me2 ChIP assay in *FLC* regions in *cdkc;2–3* and Ler. (*D*) H3Ac ChIP assay in *FLC* regions in *cdkc;2–3* and Ler. (*B–D*) Values are means ± SEM from three biological repeats; data were presented as ratio of (modified histone level at *FLC*/H3 *FLC*) to (modified histone level at *Actin*/H3 *Actin*). *Actin*, *STM*, and *IGN5* were used as internal controls for the ChIP experiments. *P < 0.05.

Table 1. Summary of mass-spectral sequencing analysis of CDKC;2-FLAG purification IP-specific peptides

Protein	Predicted molecular mass, Da	Unique (total) peptides	
		Exp. 1	Exp. 2
CDKC;2-FLAG (AT5G64960)	57,130	16 (19)	20 (28)
CYCT1;5 (AT5G45190)	65,168	5 (5)	17 (21)
CYCT1;4 (AT4G19600)	60,710	1 (1)	4 (4)

Minimum peptide identification probability value is 95%.

the *COOLAIR* promoter and its role as a positive transcriptional elongation factor. Interestingly, the CTD Ser^2 phosphorylated Pol II levels are slightly increased in the endogenous gene in the context of the linked sense–antisense transcription circuitry (Fig. 5*B* and Fig. S7*B*). Together, these data suggest that, in wild-type plants, CDKC;2 promotes transcription of *COOLAIR*, which in turn indirectly decreases *FLC* expression (Fig. 5*D*). The counterintuitive outcome is that, through feedback mechanisms likely involving the *FLC* gene loop (41), when CDKC;2 is lost and *FLC* transcription increased, there is a secondary effect to increase *COOLAIR* transcription, thus masking the direct effect of the *cdkc;2* mutations.

Discussion

The prevalence of antisense transcripts in many genomes suggests that they may have a widespread functional role (42, 43). Studies of specific loci in *Saccharomyces cerevisiae* (44) have demonstrated how sense/antisense pairs can function as genetic toggles switching expression states, but the general mechanisms in higher eukaryotes are yet to be fully established. We have been studying the sense/antisense transcriptional circuitry at the *FLC*/*COOLAIR* locus in the *Arabidopsis thaliana* genome. *FLC* encodes a floral repressor so quantitative changes in gene expression cause altered flowering time, an important adaptive trait in plants.

Our study showed that *Arabidopsis* P-TEFb transcription elongation complex promotes antisense *COOLAIR* transcription. The primary defect caused by the *cdkc;2* mutation was revealed by analysis of the expression of a *COOLAIR::LUC* fusion, which no longer expressed the *FLC* sense transcription unit. The clear reduction of *COOLAIR::LUC* expression and reduced CTD Ser^2 phosphorylated RNA Pol II occupancy at the *COOLAIR* promoter in *cdkc;2* contrasted with the lack of effect of *cdkc;2* mutations on an *FLC* transgene engineered to lack *COOLAIR* production. Thus, we conclude that, in the endogenous gene context, *cdkc;2* indirectly up-regulates *FLC* expression through disruption of a *COOLAIR*-mediated repression mechanism. Complex feedback mechanisms central to *FLC* regulation then lead to the counterintuitive outcome that, as a secondary effect, expression of *COOLAIR* increases. The feedback mechanisms that lead to the positive correlation of *FLC* and *COOLAIR* transcription remain to be fully established. However, the presence of an *FLC* gene loop involving the physical interaction of the 5′ and 3′ regions (41), and/or the antisense transcription unit fully encompassing the sense transcription unit (38), could both be involved in efficient recycling of RNA polymerase between strands.

The genetic functioning of CDKC;2 within the autonomous pathway supports the view that the repression of *FLC* expression involves a *COOLAIR*-mediated repression mechanism, in many respects rather similar to antisense-mediated repression of yeast genes (43–45). Loss of this mechanism leads to increased levels of distally polyadenylated *COOLAIR* transcripts and increased *FLC* transcription. The positive correlation between distal polyadenylation of *COOLAIR* with increased *FLC* expression has led to the suggestion that the process of antisense transcription facilitates access of *FLC* promoter sequences by the transcription machinery, so enhancing expression (24). This view was reinforced because of the concern that the absolute amount of proximal polyadenylation did not change in some autonomous pathway mutants (46). However, the higher *FLC* expression of the *FLC-TEX* transgene, which lacks the *COOLAIR* promoter and proximally polyadenylated *COOLAIR* transcript and has reduced antisense transcription (Fig. S6), is consistent with proximal polyadenylation of *COOLAIR* actively repressing *FLC* expression. The feedback mechanisms that so tightly connect sense and antisense transcription at *FLC* mean that the absolute changes in transcript level need to be interpreted carefully; *COOLAIR* transcription can drop as a primary defect in *cdkc;2* but increase in the endogenous gene context due to release of the locus-wide repression mechanism.

P-TEFb is required for transcription of most genes via its kinase activity, leading to Ser^2 phosphorylation of the RNA Pol II CTD. This posttranslational modification is associated with transcriptional elongation and intimately linked with splicing, polyadenylation, and nuclear export (30). A surprise therefore was that *COOLAIR* transcription but not sense *FLC* transcription

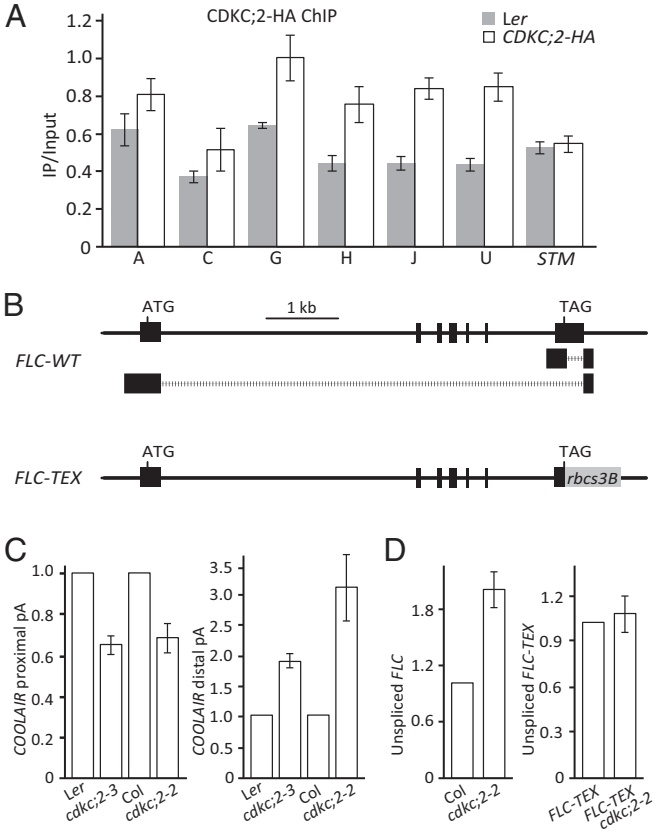

Fig. 4. CDKC;2 associates directly with the *FLC* locus and requires *COOLAIR* to repress *FLC* expression. (*A*) ChIP experiments showing enrichment of *FLC* regions in by CDKC;2-HA. The regions A, C, G, H, J, and U analyzed by qPCR are shown in the schematic in Fig. 3A. (*B*) Schematic diagram showing the replacement of the *FLC* 3′ region with that from the *Arabidopsis rbcs3B* gene to form the *FLC*-terminator exchange construct or *FLC-TEX*. (*C*) Relative use of the proximal polyadenylation site of *COOLAIR* is reduced in *cdkc;2–3* (the *Ler* allele identified in this study) and *cdkc;2–2* (the Columbia T-DNA insertion allele), and relative use of the distal site increased. The proximally and distally polyadenylated *COOLAIR* transcript levels are normalized to total *COOLAIR* transcript level and given as fold change compared with the parental lines. (*D*) Unspliced *FLC* or *FLC-TEX* transcript analysis by qRT-PCR. Values are means ± SEM from three biological repeats.

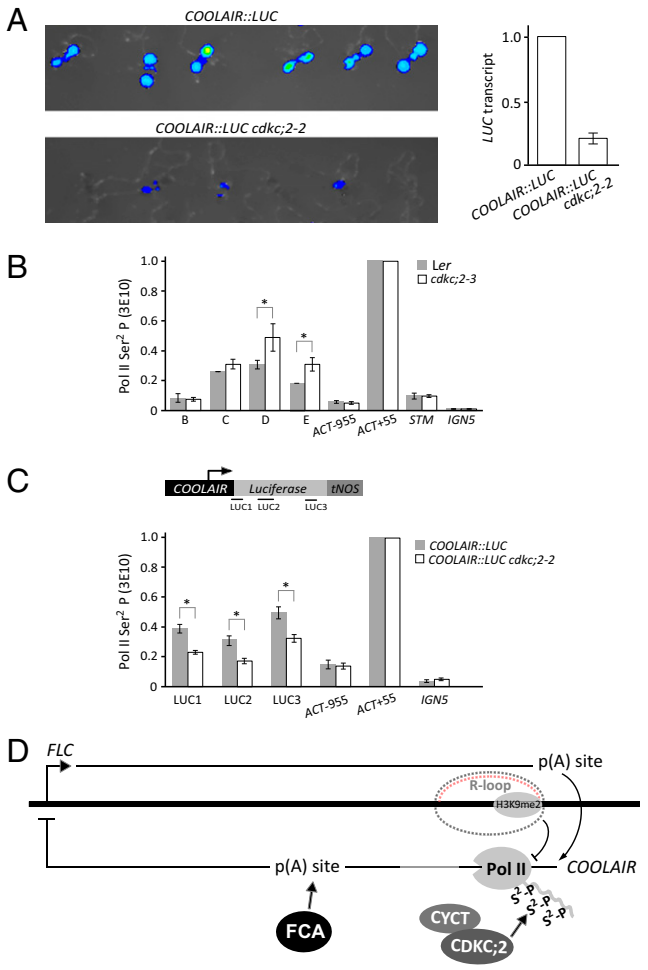

Fig. 5. CDKC;2 is required for efficient *COOLAIR* transcription. (*A*) Effects of *cdkc;2–2* on a *COOLAIR::LUC* fusion, expressed separately from the endogenous *FLC*. Luciferase activity and *COOLAIR::LUC* transcript analysis by qRT-PCR are shown. Values are means ± SEM from three biological repeats. (*B*) ChIP experiments assaying Pol II (using anti-Ser^2 P-CTD-3E10) in *FLC* regions in *cdkc;2–3* and Ler. (*C*) Schematic showing the *COOLAIR::LUC* fusion and primers used in ChIP analysis plus ChIP experiments assaying RNA Pol II (using anti-Ser^2 P-CTD-3E10) in *COOLAIR::LUC* regions in *cdkc;2–2* and Col. (*B* and *C*) Values are means ± SEM from two biological repeats; data were presented as ratio of (Pol II *FLC*/input *FLC*) to (Pol II *Actin*/input *Actin*). *Actin*, *STM*, and *IGN5* were used as internal controls for the ChIP experiments. (*D*) Working model for how CDKC;2 functions in the *FLC* sense–antisense transcriptional circuitry. CDKC;2 is a component of P-TEFb required for *COOLAIR* transcription. It is required to produce the proximally polyadenylated *COOLAIR* transcript in the FCA-dependent pathway. The proximally polyadenylated *COOLAIR* transcript represses *FLC* transcription. Feedback mechanisms link sense and antisense transcription levels so, when autonomous pathway function is compromised and *FLC* transcription increases, *COOLAIR* transcription is increased (indicated by the positive arrow on the right of the schematic). The R-loop that covers the *COOLAIR* promoter (dashed line with the RNA:DNA heteroduplex shown as a red/black pair) could make *COOLAIR* transcription more sensitive to CDKC;2 function.

was sensitive to loss of CDKC;2. Indeed, the differential sensitivity of sense and antisense transcription to this P-TEFb function appears to be an important component of the cotranscriptional mechanism limiting *FLC* expression. What causes this differential sensitivity is an interesting question. *COOLAIR* transcription initiates from a noncanonical promoter within a genomic region carrying termination sequences for the sense transcript, a feature frequently found in yeast (40). Small RNAs (24- and 30-mers) can be detected homologous to the *COOLAIR* promoter, and these are required to maintain a small patch of H3K9me2-modified chromatin just upstream of the major *COOLAIR* start site (47). This noncanonical promoter could be particularly sensitive to *CDKC;2* function to ensure Pol II promoter clearance and entry into the elongating phase of transcription and cotranscriptional maturation of the transcripts. There may therefore be a greater dependence on P-TEFb function for recruitment of the 3′ processing machinery (including CstF and CPSF complexes) to the *COOLAIR* transcript (48, 49). Any change in CstF or CPSF recruitment would influence poly (A) site choice in the antisense transcript (50) so loss of *CDKC;2* might be functionally analogous to a *cstf64* or *cstf77* mutation, previously isolated in the same suppressor screen (18). It is also possible that the 500- to 700-nucleotide R-loop, which covers the *COOLAIR* promoter and first exon and is stabilized by a homeodomain protein, AtNDX (25), could make *COOLAIR* transcription more sensitive to P-TEFb function. Enhanced cotranscriptional recruitment of splicing factors to the nascent transcript has been shown to resolve R-loops, thus aiding transcription (51).

Because P-TEFb is such a central component of transcriptional regulation, the viability of *cdkc;2* loss-of-function mutants suggests some genetic redundancy with the second copy of *CDKC*, the *CDKC;1* gene in the *A. thaliana* genome (Fig. S5*B*). However, the observed phenotypes show that the two copies cannot be fully redundant (Fig. S1 *C–F*). A division of labor in the transcriptional elongation function of P-TEFb components also seems to be the case in *S. cerevisiae* (52) and *Schizosaccharomyces pombe* (53). Recent studies demonstrating a second metazoan CTD Ser^2 kinase (54, 55) suggest that the paradigm of CTD Ser^2 kinase pairs might be retained throughout higher eukaryotes. The *A. thaliana* proteins CDKC;2 and CDKC;1 display CTD kinase activity in vitro (26), are associated with cyclin T (CycT), and have a conserved CTD kinase domain so it is likely that expression differences determine their division of labor.

In conclusion, our data reveal the complexity in the sense–antisense transcriptional circuitry at the *Arabidopsis FLC* locus. Sensitivity of *COOLAIR* transcription to P-TEFb function is an important component of the cotranscriptional mechanism limiting *FLC* expression. Loss of CDKC;2 reduces *COOLAIR* production, which disrupts the restraint on sense transcription. Through mechanisms that link sense–antisense transcription, probably involving kinetic coupling of chromatin state, transcription elongation rate, and RNA processing, in combination with gene loops, this increased *FLC* transcription then feeds back to increase *COOLAIR* transcription. These kinds of cotranscriptional mechanisms, which work *in cis* only, are clearly not limited to plant genomes (56, 57). Modulation of antisense transcription by P-TEFb linked to gene body chromatin modification could be a general mechanism regulating gene expression in eukaryotes.

Materials and Methods

The progenitor line C2 is described in ref. 18. For the luciferase screening, *Arabidopsis* seedlings were grown on germination medium agar plates at 23–25 °C with a 16-h photoperiod for 12 d. Seedlings were sprayed with 1mM of luciferin (Promega) solution and incubated in the dark at room temperature for 25 min. The LUC bioluminescence signal of the seedlings was assayed using a Nightowl light-sensitive CCD camera (Berthold Technologies). *Arabidopsis* CDKC;2 T-DNA insertion line (SALK_029546, *cdkc;2–2*) was obtained from the Nottingham *Arabidopsis* Stock Centre.

The *COOLAIR::LUC* line has been described previously and is in the *FRI flc-2* background (25). Here, the *COOLAIR::LUC* gene was introgressed into *cdkc;2–2* by crossing and genotyping. The resulting *COOLAIR::LUC cdkc;2–2* genotype contains the endogenous active *FLC* allele.

Complementation analysis, *FLC-TEX* cloning, expression analysis using real-time RT-PCR, measurement of the *COOLAIR* levels in different genetic backgrounds, ChIP assays, Western blotting, CDKC;2 purification for proteomic analysis, and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis are all described in *SI Materials and Methods*. Primers are listed in Table S1.

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