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Functional Consequences of Splicing of the Antisense Transcript COOLAIR on FLC Transcription

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SUMMARY

Antisense transcription is widespread in many genomes; however, how much is functional is hotly debated. We are investigating functionality of a set of long noncoding antisense transcripts, collectively called COOLAIR, produced at Arabidopsis FLOWERING LOCUS C (FLC). COOLAIR initiates just downstream of the major sense transcript poly(A) site and terminates either early or extends into the FLC promoter region. We now show that splicing of COOLAIR is functionally important. This was revealed through analysis of a hypomorphic mutation in the core spliceosome component PRP8. The prp8 mutation perturbs a cotranscriptional feedback mechanism linking COOLAIR processing to FLC gene body histone demethylation and reduced FLC transcription. The importance of COOLAIR splicing in this repression mechanism was confirmed by disrupting COOLAIR production and mutating the COOLAIR proximal splice acceptor site. Our findings suggest that altered splicing of a long noncoding transcript can quantitatively modulate gene expression through cotranscriptional coupling mechanisms.

INTRODUCTION

The biological significance of non-protein-coding genomic sequences has been an issue for decades (Britten and Davidson, 1969; Mattick, 2004). This has recently been reinforced by the finding that most of the human genome is represented in primary transcripts (Djebali et al., 2012). The majority of these are long, spliced, and polyadenylated RNA Polymerase II (RNA Pol II) transcripts, and a large number are antisense transcripts to annotated genes (Derrien et al., 2012; Osato et al., 2007; Lehner et al., 2002; Lu et al., 2012; Wang et al., 2005; Yamada et al., 2003). Many of the long (>200 nt) noncoding RNAs show no evolutionary conservation, adding to the debate of whether they serve any function (Gerstein et al., 2012; Graur et al., 2013).

Several in-depth studies in yeast have shown that noncoding transcripts have the potential to regulate gene expression through transcriptional interference or recruitment of chromatin modifiers (Camblong et al., 2007; Hongay et al., 2006; Castelnuovo et al., 2013). However, roles of noncoding transcripts in higher eukaryotes are less well understood. Some have been shown to play roles in chromatin regulation (Wang and Chang, 2011), although it can be the transcriptional overlap rather than the antisense transcript itself that is important for the functional consequence (Latos et al., 2012).

We have focused on the functional consequences of antisense transcription through our study of the regulation of Arabidopsis FLOWERING LOCUS C (FLC) gene, a developmental regulator that controls the timing of the switch to reproductive development. FLC encodes a MADS box transcriptional regulator that represses flowering, and FLC expression quantitatively correlates with flowering time (Sheldon et al., 1999; Michaels and Amasino, 1999). There are several regulatory pathways that converge to regulate FLC: two that antagonistically regulate FLC in ambient temperatures—the FRIGIDA pathway, which activates FLC expression, and the autonomous pathway, which downregulates FLC—and one more, vernalization, which epigenetically silences FLC in response to prolonged cold (Figure 1A). All of these pathways involve a set of antisense transcripts, collectively named as COOLAIR, that fully encompass the FLC gene, initiating immediately downstream of the sense strand polyadenylation site and terminating beyond the sense transcription start site (Hornyik et al., 2010; Liu et al., 2010; Swiezewski et al., 2009). COOLAIR transcripts are polyadenylated at multiple sites with proximal polyadenylation promoted by components of the autonomous promotion pathway. These include the RNA-binding proteins FCA and FPA, the 3′ processing factors Cstf64, Cstf77 and FY, the CPSF component and homolog of yeast Pfs2p and mammalian WDR33 (Liu et al., 2010; Ohnacker et al., 2000; Simpson et al., 2003). Use of the proximal poly(A) site results in quantitative downregulation of FLC expression in a process requiring FLD, an H3K4me2 demethylase (Liu et al., 2010). FLD activity results in H3K4me2 demethylation in the gene body of FLC and transcriptional downregulation of FLC (Liu et al., 2007, 2010). Loss of any of the autonomous pathway components reduces usage of the proximal polyadenylation site, which leads to increased FLC transcription. Analysis of the regulation of COOLAIR transcription has recently identified an RNA-DNA heteroduplex, or R-loop, covering the COOLAIR promoter (Sun et al., 2013). Stabilization of this R-loop by a novel homeodomain protein limits
COOLAIR transcription, adding another layer of regulation within the autonomous pathway.

We have continued to investigate the transcriptional circuitry at FLC and how COOLAIR is linked to changes in FLC expression. Here, through identification of a hypomorphic mutation in the core spliceosome component PRP8, we reveal how COOLAIR functionally modulates FLC gene expression through a cotranscriptional coupling mechanism. The prp8 mutation reduces splicing efficiency of COOLAIR introns and usage of the proximal poly(A) site, increasing histone methylation in the gene body and upregulating FLC transcription. We also show a positive feedback mechanism between gene body histone methylation and COOLAIR processing. The involvement of COOLAIR splicing in this mechanism was supported through both disruption of COOLAIR production and cis mutation of the antisense proximal splice acceptor site. Cotranscriptional coupling mechanisms such as this may be of widespread importance in the quantitative regulation of gene expression.

RESULTS
A Hypomorphic Mutation in the Core Splicing Factor PRP8 Affects FLC Expression

We pursued a suppressor mutagenesis strategy to identify additional factors contributing to flowering time regulation through FLC repression by FCA (Figure 1A). We mutagenized a line that is suitable to identify factors required for FCA-mediated FLC repression (also referred to as C2; Liu et al., 2010). It relies on FCA overexpression (35S-FCAγ transgene) to enhance FCA activity and establish low levels of FLC, a FLC-LUCIFERASE (FLC-LUC) reporter to efficiently monitor FLC levels, and a functional FRIGIDA (FRI) allele to amplify changes in FLC expression to increase sensitivity of detection (Johanson et al., 2000). Interestingly, the commonly used Arabidopsis accessions such as Landsberg erecta (Ler) and Columbia (Col) contain loss-of-function fri alleles, and the functional FRI we added originated from a Swedish accession (Johanson et al., 2000).

We screened for mutants with increased luciferase activity of FLC-LUC and identified suppressor of overexpressed FCA (sof) 81 (Figure 1B). sof81 was a weaker suppressor than fld, the first mutant identified as a sof (Liu et al., 2007), and was found to be recessive in crosses to the C2 progenitor (Figure 1B). The mutation was mapped to At1g80070 (Figures S1A and S1B available online), a gene that has previously been identified as essential for plant development, as null mutations lead to embryonic lethality and abnormal suspensor development (sus phenotype) (Schwartz et al., 1994). At1g80070 encodes PRP8, the conserved and central component of the spliceosome (Grainger and Beggs, 2005). The sof81 mutation changes a glycine to glutamic acid at amino acid position 1,891 (Figure 1C) within the RNase H domain of PRP8 (Figure 1D). The mutation did not change PRP8 protein levels in the plant (Figure S1C). The RNase H domain of PRP8 is thought to be an integral part of the spliceosome (Pena et al., 2008; Galej et al., 2013) that prevents premature U4/U6 unwinding and acts as a platform for exchange of U6 snRNA for U1 at the 5’ splice site (Mozaffari-Jovin et al., 2012).

The five available null alleles of PRP8 (sus2) plants are embryonic lethal, indicating the mutation in sof81 (referred to from now on as prp8-6) is hypomorphic. The prp8-6 mutant phenotype was rescued by a genomic PRP8 clone (Figure S2A), and heteroallelic combinations between one copy of a prp8-sus2 allele (either sus2-4 or sus2-5) and one copy of the prp8-6 allele showed no complementation based on FLC-LUC bioluminescence and flowering-time analyses (Figures S2B and S2C). We therefore conclude that prp8-6 is a recessive, hypomorphic mutation that increases FLC expression in sof81. Unlike yeast and human, Arabidopsis thaliana carries a second copy of PRP8 (Atg38780) transcribed at low levels (Figure S1D) (Liu et al., 2009); however, given the mutant phenotype, this cannot completely cover the function of At1g80070 in FLC regulation.

The prp8 Mutation Also Affects Endogenous FLC Expression and Flowering Time

A similar forward mutagenesis screen had led to identification of DCL4 as a regulator of FCA expression with reduction in FCA expression resulting in elevated levels of FLC (Liu et al., 2012). Therefore, we first tested whether there was any change in the expression or functionality of FCA in prp8-6. We found no change in expression of the transgene 35S-FCAγ by western and northern blot analysis (Figures S3A and S3B). Additionally, the autoregulatory feedback limiting FCA levels was unaffected (Figures S3B and S3C) (Quesada et al., 2003). Previous data had shown that FCA associates with FLC chromatin (Liu et al., 2007). We found no reduction of FCA binding to the FLC locus.

Figure 1. sof81 Is a Mutation in the Splicing Factor PRP8 that Disrupts FLC Expression

(A) The different pathways that antagonistically regulate FLC expression are shown.

(B) Mutant screening. Three representative Arabidopsis seedlings of sof81, the C2 progenitor, and F1 individuals from sof81 or C2 crossed to Columbia are shown. False-colored FLC-LUCIFERASE bioluminescence activity is superimposed onto the seedlings.

(C) Sequence alignment of the conserved PRP8 RNase H domain region containing the G1891E mutation in prp8-6. At, Arabidopsis thaliana; Hs, Homo sapiens; Sc, Saccharomyces cerevisiae.

(D) A schematic representation of the PRP8 protein. Domains and positions of different mutations are shown. Yellow box indicates the epitope of hPRP8 antibody BMR 00434 (Figure S1C). See also Figures S1 and S2.
in prp8-6; if anything, there was an elevated level (Figure S3D). A similar lack of effect of prp8-6 was observed on expression of other autonomous pathway components (Figure S3E); thus, we concluded that the increase of FLC expression by prp8-6 is unlikely to be due to an indirect effect on autonomous pathway function.

Various polymorphisms have been reported between the FLC alleles of the Col and Ler laboratory strains (Col-FLC and Ler-FLC), including the presence of a Mutator transposon at the 3’ end of intron 1 (Liu et al., 2004). As FLC-LUC is based on Col-FLC, we tested the effect of prp8-6 on both alleles in the same samples by northern blotting using an FLC probe that discriminates by size. We detected only two transcript species reflecting FLC samples by northern blotting using an FLC probe that discriminates by size. We detected only two transcript species reflecting FLC

We then undertook an extensive genetic study analyzing combinations of fca-1, prp8-6, and FRIGIDA to investigate how PRP8 influences the autonomous and FRIGIDA pathway (Figure 2C). prp8-6 delayed the early flowering of the progenitor line (carrying the 35S::FCA-γ and FRIGIDA transgenes) and delayed flowering much more extensively when the 35S::FCA-γ transgene was crossed out, but was epistatic (nonadditive) with the loss-of-function mutation of FCA, fca-1. Consistent with this, when prp8-6 was combined with just FRI, the expression of FLC was significantly higher (Figure 2D). The effect of prp8-6 in fca genotypes increased when in combination with a sus2 null allele suggesting stronger alleles than prp8-6 would confer later flowering if they were viable (Figures 2C and S2C). The epistasis (nonadditivity) of prp8-6 with fca-1 indicates that PRP8 works in the same genetic pathway as FCA in wild-type plants. Overall, these results suggest the prp8-6 mutation causes a small reduction in PRP8 activity, which functions in the same genetic pathway as FCA to oppose FRIGIDA activation of FLC.

PRP8 Influences Sense FLC Expression through Effects on COOLAIR Splicing

PRP8 has a central role in splicing in most eukaryotes, but since the null phenotype is embryonic lethality (Schwartz et al., 1994), a role in FLC regulation had not previously been detected. Interestingly, mutations in two other Arabidopsis splicing factors, SR45 and PRP39-1, have been shown to increase FLC expression and cause late flowering (Ali et al., 2007; Wang et al., 2007). We therefore analyzed the effect of prp8-6 on splicing of FLC transcripts and also more generally. Four alternatively spliced gene models are annotated for sense FLC (Figure 3A). Measuring splicing efficiency of these alternative FLC introns by quantitative RT-PCR (qRT-PCR) indicated no effect of prp8-6 on these splicing events (Figure 3B). No disruption of the size of the sense transcript was detected by northern blot analysis, even though a cis mutation in an FLC sense splice site in the flc-6 mutant reveals this species (Figure S4A). The splicing efficiency of two control genes was also unaffected in prp8-6 (UBC9 and EF1a, Figure 3C).

As our analysis provided no evidence of FLC sense splicing defects we analyzed splicing of COOLAIR introns. COOLAIR is alternatively spliced in different environmental conditions and different genotypes (Hornyik et al., 2010; Liu et al., 2010; Siewezewski et al., 2009). We assayed the efficiency of splicing of introns present in the most abundant COOLAIR transcripts (schematically summarized in Figure 3A) and found it was reduced in prp8-6 (Figures 3D). The class III and IV forms represent <1% of the COOLAIR transcripts and so were not analyzed (Hornyik et al., 2010). There was no similar reduction in splicing efficiency of the class II intron in a mutant of another splicing regulator, PRP39 (Figure S4B), showing that not all splicing regulators influence FLC expression through the same mechanism. We explored the consequences of the prp8-altered splicing efficiency on the poly(A) site choice in the COOLAIR transcript. Alternative poly(A) sites clustered in proximal and distal regions have been characterized (Hornyik et al., 2010; Liu et al., 2010; Siewezewski et al., 2009). We used a qRT-PCR assay that specifically monitored one of the proximal and one of the distal poly(A)
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Figure 3. prp8-6 Influences Splicing Efficiency of COOLAIR Introns
(A) Schematic representation of transcripts from the FLC locus. FLC and COOLAIR isoforms are shown including positions of primers (gray arrows and lines) used to determine splicing efficiencies. Black rectangles denote exons. The asterisk indicates the position of the flic-6 splice acceptor cis element mutation of intron 2 (AG to AA) (Greb et al., 2007). Class ii and class iii are abundant COOLAIR isoforms in seedlings grown at warm temperatures, and class li and iii are most abundant in cold-grown seedlings (Swiezewski et al., 2009; Liu et al., 2010; Hornyk et al., 2010).

(B) Splicing of alternative FLC sense introns is not affected by prp8-6. FRI in black, and FRI prp8-6 in gray. Levels of spliced and unspliced alternative introns in FLC sense variants FLC.1-4 were determined by qRT-PCR. Splicing efficiencies (spliced/unspliced) are given normalized to FRI background control. Details of the assays are given in extended experimental procedures in the Supplemental Information.

(C) Splicing efficiency of introns in UBC and EF1a transcripts assayed using RT-qPCR is not affected by prp8-6 (gray), normalized to the FRI background control (black).

(D) Splicing efficiency of COOLAIR transcripts assayed using qRT-PCR of RNA from C2 (black) and C2 prp8-6 mutants (gray). (B–D) Values are means ±SEM (n = 3); Student’s t test was performed. p values <0.05 are denoted by (*). See also Figures S3 and S4.

sites (primers shown in Table S1). prp8-6 reduced usage of the COOLAIR proximal poly(A) site and promoted use of the distal site (Figure 4A). Northern blot analysis showed these data are representative of poly(A) site usage of COOLAIR transcripts generally (Figure S4C).

Importance of COOLAIR Splicing in PRP8-Dependent Repression of FLC Transcription
In order to explore the role of altered COOLAIR splicing on the FLC transcriptional repression mechanism, we generated an FLC gene where expression and splicing of COOLAIR was disrupted. The 3’ region of FLC, from the translation stop site to ~700 bp downstream, was exchanged with that from Arabidopsis gene rbc3B (At5g38410). This generated an FLC transgene (named FLCex) that encoded a sense transcript with a different 3’ UTR and lacked the COOLAIR promoter, exon 1, and intron 1. The FLCex and wild-type FLC constructs were transformed into a loss-of-function FLC genotype (FRI flic-2), which has a deletion/ rearrangement within the endogenous FLC gene (Michaels and Amasino, 1999). FLC transcription (assayed as spliced transcript accumulation) was ~3-fold higher in FLCex lines compared to FLC transgenic lines (Figure 4B). We crossed prp8-6 into three independent, representative lines carrying the FLCex of FLC transgenes and assayed FLC expression (Figure 4B). This enabled us to compare the effect of prp8-6 on individual transgene insertions and avoid the issue of between transgenic line expression variability. prp8-6 did not lead to any further increases in expression in combination with FLCex. This epistasis is consistent with loss of COOLAIR production and prp8-6 influencing the same mechanism.

Since reduced use of the COOLAIR proximal poly(A) site disrupted transcriptional repression of FLC (Liu et al., 2010), we reasoned that reduced splicing efficiency of the COOLAIR class li intron, necessary to generate the exon containing that poly(A) site, might be an important factor in the increased expression of FLC in prp8. We therefore specifically blocked splicing of this intron by site-directed mutagenesis of the terminal intronic dinucleotide AG to AA (Figure 5A). Mutation at this site has a minimal effect on the sense transcript, introducing one nucleotide change to the 3’ UTR downstream of the FLC open reading frame. Multiple, independent transgenic lines containing either wild-type FLC or FLC carrying the AG-to-AA mutation (COOLAIRAA) were generated in a FRI flic-2 genotype, each with and without the prp8-6 mutation, and analyzed in pools. The AG-to-AA mutation (COOLAIRAA) significantly reduced splicing efficiency of the intron and increased levels of FLC expression (Figure 5B). When combined with prp8-6, the AG-to-AA mutation did not further increase FLC levels relative to prp8-6 alone (Figure 5B), suggesting that at least some of the prp8-6 phenotype is the result of altered splicing of the class li
intron. Often when the AG dinucleotide at the end of an intron is mutated downstream, AG dinucleotides are utilized instead. We used PCR with flanking primers, but we did not detect other splicing events (Figure S6A). Proximal poly(A) site usage of COOLAIR was reduced, and this was not additive to the prp8-6-induced changes (Figures 5B). Overall, these data support the view that the prp8-6 phenotypic effects are smaller than many other autonomous pathway mutants but involve reduced splicing of COOLAIR class II intron, which reduces COOLAIR proximal poly(A) site usage.

**Coupling of Splicing, Chromatin State, and Transcriptional Level**

Alternative polyadenylation of the COOLAIR transcripts has been shown to trigger changes in histone methylation, increased transcription as assayed by unspliced transcript production, and RNA Pol II occupancy at the FLC locus (Liu et al., 2007, 2010). We therefore analyzed whether prp8-6 influenced H3K4 demethylation and Pol II occupancy at FLC. prp8-6 increased H3K4me2 in the body of the gene downstream of the proximal COOLAIR poly(A) site (Figures 6A and S6B), similar to changes
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Figure 6. Coupling between PRP8 Function, Autonomous Pathway Activity, and Histone Demethylation at FLC

(A) Chromatin immunoprecipitation (ChIP) assays to analyze H3K4me2 enrichment at FLC. C2 prp8-6 (gray) values are normalized to C2 control (black). Schematic representation of FLC with black rectangles denoting exons. Horizontal bars in A, C, G, and H denote regions analyzed by qPCR in ChIP experiments. H3K4me2 levels in regions A, G, and H are significantly higher in prp8-6 than in C2 (p < 0.05).

(B) Pol II occupancy in different regions of FLC in C2 prp8-6 (gray) normalized to the C2 control (black). PolII levels in all regions are significantly higher in prp8-6 than in C2 (p < 0.05).

(C and D) Splicing efficiency of COOLAIR introns is affected by fca (gray) and fld (white). Averages are from three biological repeats ±SEM in fca-1 and fld-6 normalized to Ler wild-type.

(E and F) Relative usage of a proximal poly(A) site of COOLAIR is reduced, and a distal poly(A) site increased in fca and fld mutants. Proximal and distal poly(A) site usage is expressed as relative to total COOLAIR. Averages are from three biological repeats ±SEM in fca-9 and fld-4 normalized to Col wild-type.

(Figure S7B–S7D)

DISCUSSION

The functional importance of long noncoding RNAs is a major issue in molecular biology. Analysis of the control of flowering time has enabled us to address this issue by investigating the roles of a set of long noncoding transcripts, collectively called COOLAIR, produced at the Arabidopsis locus FLC. FLC encodes a repressor of flowering whose expression level determines whether plants over-winter before flowering. Here, analysis of a hypomorphic mutation in the essential PRP8 spliceosomal subunit suggests a role for COOLAIR splicing in the quantitative modulation of FLC transcription. This hypomorphic mutation is likely to reveal the sensitivity of FLC regulation to changes in general function gene regulators, rather than particular specificity in PRP8 targets. Genetic and molecular analysis revealed that

induced by fld and fca mutations (Liu et al., 2007). We addressed whether these changes were mediated through FLD, the H3K4me2 demethylase involved in FLC downregulation. Consistent with a connection between PRP8 activity and FLD-induced H3K4me2 demethylation, we found that combination of the hypomorphic prp8-6 allele with a weak fld mutation led to a synergistic effect on FLC derepression (Figure S7A). As with fca and fld mutants, the increase in H3K4me2 in prp8-6 was associated with increased PolII occupancy (Figures 6B, S6C, and S6D). These data support a model whereby efficient splicing of class II intron via PRP8 activity promotes proximal poly(A) site choice in the antisense transcript via FCA, FY activity. In turn, this proximal polyadenylation triggers FLD-mediated H3K4me2 demethylation in the gene body, which represses transcription of FLC.

We then investigated how splicing and polyadenylation of COOLAIR might be coupled with the chromatin state at FLC in two ways. First, we analyzed COOLAIR splicing and polyadenylation in the fld demethylase mutant. The splicing efficiency of antisense introns class II was significantly reduced in fld, as in fca (Figures 6C and 6D). In addition, proximal poly(A) site usage was reduced (Figure 6E) and distal poly(A) site usage increased (Figure 6F) in an fld mutant. This suggested that there was positive feedback between the chromatin state at FLC and alternative COOLAIR splicing and polyadenylation. Second, we analyzed seedlings treated with the histone deacetylase inhibitor trichostatin A in order to increase the acetylation level of FLC chromatin. This was stimulated by the observation that fld mutations result in hyperacetylation of histones in FLC chromatin (He et al., 2003). As expected, transcriptional activity at the locus assayed by FLC unspliced RNA increased (Figure 7A). This was associated with an increase in total COOLAIR production (Figure 7B), consistent with previous data of a positive correlation between total FLC and total COOLAIR production (Szwierczynski et al., 2009), and a relative reduction in proximally polyadenylated COOLAIR (Figure 7C). This further supported a positive feedback mechanism coupling chromatin state with COOLAIR processing. Chromatin modification has been proposed to affect transcript processing indirectly through influencing transcription elongation rate (Allolio et al., 2009). If this is the case here, it is not dependent on the transcriptional pause release factor TFIIS (Grasser et al., 2009), because tflIIs mutations do not influence COOLAIR poly(A) site choice (Figures S7B–S7D).

Figure 6. Coupling between PRP8 Function, Autonomous Pathway Activity, and Histone Demethylation at FLC

(A) Chromatin immunoprecipitation (ChIP) assays to analyze H3K4me2 enrichment at FLC. C2 prp8-6 (gray) values are normalized to C2 control (black). Schematic representation of FLC with black rectangles denoting exons. Horizontal bars in A, C, G, and H denote regions analyzed by qPCR in ChIP experiments. H3K4me2 levels in regions A, G, and H are significantly higher in prp8-6 than in C2 (p < 0.05).

(B) Pol II occupancy in different regions of FLC in C2 prp8-6 (gray) normalized to the C2 control (black). PolII levels in all regions are significantly higher in prp8-6 than in C2 (p < 0.05).

(C and D) Splicing efficiency of COOLAIR introns is affected by fca (gray) and fld (white). Averages are from three biological repeats ±SEM in fca-1 and fld-6 normalized to Ler wild-type.

(E and F) Relative usage of a proximal poly(A) site of COOLAIR is reduced, and a distal poly(A) site increased in fca and fld mutants. Proximal and distal poly(A) site usage is expressed as relative to total COOLAIR. Averages are from three biological repeats ±SEM in fca-9 and fld-4 normalized to Col wild-type.

(C–F) Student’s t test was performed. p values <0.05 are denoted by (*). See also Figures S6 and S7.
The effects of FRIGIDA promoting FLC expression and reduces use of the proximal COOLAIR polyadenylation site. Arabidopsis seedlings (C2 genotype) were treated with (black) or not (white) with TSA. qPCR data were normalized to UBC. Averages are from three biological repeats ±SEM. All comparisons are statistically significant at p < 0.05.

(D) The autonomous pathway promotes use of the proximal antisense splice acceptor site and increases relative use of the antisense proximal poly(A) site. This results in FLD-dependent H3K4me2 demethylation in the gene body of FLC. We envisage that the chromatin modifications influence transcription elongation rate of both strands, leading to low FLC expression and reinforcing the choice of the proximal splice acceptor site of the class II intron and proximal polyadenylation of COOLAIR.

Figure 7. Cotranscriptional Coupling of Chromatin Modification with COOLAIR Processing

(A–C) Inhibition of histone deacetylase activity by trichostatin A (TSA) increases FLC expression and reduces use of the proximal COOLAIR polyadenylation site. Arabidopsis seedlings (C2 genotype) were treated (white) or not (black) with TSA. qPCR data were normalized to UBC. Averages are from three biological repeats ±SEM. All comparisons are statistically significant at p < 0.05.

(D) The autonomous pathway promotes use of the proximal antisense splice acceptor site and increases relative use of the antisense proximal poly(A) site. This results in FLD-dependent H3K4me2 demethylation in the gene body of FLC. We envisage that the chromatin modifications influence transcription elongation rate of both strands, leading to low FLC expression and reinforcing the choice of the proximal splice acceptor site of the class II intron and proximal polyadenylation of COOLAIR.

PRP8 functions in the autonomous pathway. This pathway represses FLC expression via promotion of COOLAIR proximal polyadenylation associated with gene body histone methylation changes and lower transcription. The hypomorphic mutation in PRP8 reduces the splicing efficiency of COOLAIR introns, reducing proximal polyadenylation and autonomous pathway function. The similar molecular phenotypes of the components of the autonomous pathway with respect to splicing, polyadenylation, and chromatin modification point to a positive feedback mechanism via cotranscriptional coupling between the chromatin methylation in the gene body and processing of the COOLAIR transcript (Figure 7D).

The prp8-6 amino acid substitution is the first instance in which development of higher organisms is influenced by changes in the RNase H domain of PRP8. The viability of the mutant plants and the lack of effect on the splicing efficiency of sense FLC introns or other control transcripts argues for this substitution, causing only a slight reduction in PRP8 function. The effects of FRIGIDA promoting FLC transcription would enhance this small impairment of the autonomous pathway repression. Interestingly, specific developmental defects have been identified previously for mutations in other regions of PRP8. Retinitis pigmentosa (RP) is a heritable human disease that describes progressive degeneration of the retina during development, leading to blindness; one of the heterogeneous causes of RP is a set of mutations all clustering to the C terminus of PRP8 (Liu and Zack 2013). While the disease mechanism of RP-associated mutations in PRP8 is not fully understood, our findings suggest that a sensitivity to PRP8 may arise through cotranscriptional feedback regulation of retina regulators, particularly those associated with alternatively spliced noncoding transcripts. Exactly how the G1891E substitution impairs splicing is unknown, as is the close connection between COOLAIR intron 1 splicing and choice of proximal poly(A) site. Autonomous pathway mutations may be useful in the dissection of the tight connection between poly(A) site choice and last intron acceptor site choice (Martinson, 2011). Read-through transcription occurs in the A. thaliana genome when autonomous pathway function is impaired (in fcafpΔ double mutants) (Sonmez et al., 2011); however, these read-through products are generally spliced, resulting in poly(A) sites remaining relatively close to 3′ acceptor sites. An important aspect of our work here has been the elaboration of the cotranscriptional coupling between COOLAIR and FLC transcription. Analysis of fld, the histone K4 demethylase mutant (Liu et al., 2007), and trichostatin A treatment suggested a positive feedback mechanism coupling gene body histone methylation with COOLAIR splicing and polyadenylation. Chromatin modifications have previously been shown to mediate alternative splicing (Batschê et al., 2006; Saint-André et al., 2011; Kornblihtt et al., 2013), but less is known about how alternative splicing induces chromatin changes. In the case of FLC, alternative processing of COOLAIR leads to histone methylation changes in the gene body, in an as yet undefined mechanism, but this coupling provides a positive feedback loop reinforcing splicing and chromatin modification outcomes. We envisage a feedback loop functioning via a kinetic coupling mechanism as proposed by Allé et al., (2009). A low expression state promoted by the autonomous pathway would be characterized by use of the proximal COOLAIR splice acceptor site, increased proximal polyadenylation, and FLD-dependent H3K4me2 demethylation. This state would be maintained through positive feedback with low H3K4 methylation, reinforcing use of the proximal splice acceptor site. Reduced RNA Pol II elongation rate is a likely component of this loop, as slow transcription has been linked to proximal splice site choice and early termination (de la Mata et al., 2003; Hazelbaker et al., 2013). Feedback mechanisms may generally link transcriptional elongation and alternative splicing with changed polyadenylation. For example, in the case of IgH, increased transcriptional elongation leads to read-through at a weak splice acceptor site and results in proximal polyadenylation (Martincic et al., 2009). Lariat-derived circular intronic long noncoding RNAs (ciRNAs) have recently been isolated from the nonpolyadenylated RNA population in human cells and shown to promote Pol II transcription (Zhang et al., 2013). It will be interesting to investigate if such RNAs are important in the interplay between COOLAIR isoforms and Pol II transcription.

Feedback mechanisms tend to produce bistable systems, as clearly demonstrated by the phenotypic heterogeneity induced through metastable epigenetic toggles in yeast cell populations (Bumgarner et al., 2012). An interesting next question is whether FLC exists in alternative expression states due to changes in autonomous pathway regulation. Variation in expression of the
FLC regulators both developmentally and environmentally has previously been documented. For example, one of the components of the autonomous pathway, FCA, is itself subject to negative autoregulation via alternative polyadenylation with maximal expression in the shoot and root apical meristem not reached until 5 days after germination (Macknight et al., 1997). Temperature influences several of the autonomous and FRIGIDA pathway functions (Jung et al., 2012; Blázquez et al., 2003). All these influences could then modulate the dynamics of the feedback loop so quantitatively modulating FLC transcription. The cotranscriptional mechanism regulating expression of the floral repressor gene FLC is revealing concepts of general importance to gene regulation.

**EXPERIMENTAL PROCEDURES**

**Trans-Complementation of soфа1 with the Genomic PRP8**

The genomic region encompassing the PRP8/SUS2 gene on Arabidopsis chromosome I was inserted into a TAC library cosmide clone (pJATY50P17) that was available through the John Innes Genome Centre. A 10 kb genomic PRP8 region was amplified by PCR with the oligonucleotides PRP8-SacII-SfiI-F and PRP8-KpnI-R using pJATY50P17 as template with Phusion DNA polymerase (NEB). The PCR fragment was cloned into the binary plant transformation vector pCAMBIA-1300, containing hygromycin resistance in plants via SfiI/KpnI cloning to generate ASM4. The cloned genomic PRP8 region in ASM4 was sequenced to verify the absence of mutations. ASM4 was transformed into soфа1 mutants by Agrobacterium mediated floral-dip transformation, and hygromycin-resistant T1 transformants were isolated (n > 10). The activity of the FLC-LUC reporter of the transformants was compared to untransformed soфа1 mutant controls.

**Cloning of FLC, COOLAIRAA, and COOLAIRTEX**

FLC was cloned as a genomic SacI fragment (12 kb) into the Arabidopsis binary vector pCAMBIA-1300, which confers hygromycin resistance in plants. To generate COOLAIRAA, fragments F1 (1,250 bp) and F2 (311 bp) were amplified from FLC with primers for F1 (FLC33sF1-forward and FLC33sF1-reverse) and F2 (FLC33sF2-forward and FLC33sF2-reverse) containing a mutated sequence for the 3’ splice site of FLC antisense class II intron (AA instead of AG). PCR amplification was performed with Phusion polymerase (NEB). Resulting fragments F1 and F2 with overlapping ends were fused together in 1:1 molar ratio by PCR amplification with Phusion polymerase (NEB) employing the forward primer for F1 and the reverse primer for F2. The resulting fragment was digested with Nhel and BglII, gel purified, and subsequently cloned into an SphI fragment of FLC, replacing the wild-type Nhel-BglII fragment. The resulting SphI fragment with the mutated class II antisense 3’ splice site was inserted into FLC-pCAMBIA-1300. This mutation creates a recognition site for Dral (TTTAAA), which has been used for genotyping the hygromycin resistant transformants to verify presence of the COOLAIRAA mutation.

F2 homozygotes of the following genotypes: ppr8-6/flc-2/FRI and FRRPP8/ff-2/FRI were obtained from crosses of ppr8-6 and ff-2/FRI. The F2 homozygotes were transformed using Agrobacterium-mediated transformation of floral buds with the either FLC-pCAMBIA-1300 or COOLAIRAA-pCAMBIA-1300. The seeds from a total of 49 T1 (first generation) transformants (13 plants of COOLAIRAA/pCAMBIA-1300, 11 plants of COOLAIRAA/prp8-6/ff-2/FRI, 15 plants of FLC/FLCPR8/ff-2/FRI, and 10 plants of FLC/pCam/prp8-6/ff-2/FRI) were sown on GM medium without glucose and selected for hygromycin resistance (T2 generation). RNA for analysis was extracted from 4-week old seedlings.

For cloning COOLAIRTEX, the sequence TAGCCACC that contains FLC translational stop TAG codon was mutated to create Ehel restriction site TGGGGGCC. A SspI-SspI fragment containing the strong RBS terminator (706 bp) was PCR amplified and cloned in sense direction between Ehel and SspI restriction sites (SspI is located 741 bp downstream of the FLC stop codon, therefore replacing the corresponding genomic sequence of 3’ UTR of FLC and flanking downstream region to create COOLAIRTEX.

To analyze the effect of FLCtex transgenes were collected from four homozygous plants of FLCtex/flc-2/prp8-6/FRI and five homozygous plants of FLCtex/flc-2/PRP8/FRI. These plants were obtained from the three independent crosses of FLCtex/flc-2/FRI to prp8-6/FRI. As a control for the FLCtex analysis, the flc-2/ FRI plants were transformed with pSLJ-FLC15 (10 kg clone of Columbia FLC gene) and crossed with prp8-6/FRI (two independent crosses). Three plants from either FLC/flc-2/prp8-6/FRI or FLC/flc-2/PRP8/FRI were obtained. The seedlings from FLCtex and corresponding pSLJ-FLC transgenic plants were grown on GM medium without glucose and BASTA resistant transformants were isolated for analysis.

**Measuring FLC Sense Transcript**

For the sense FLC mRNA analysis, reverse transcription was performed using FLC specific reverse primers with SuperScript III Reverse Transcriptase (Invitrogen). qPCR analysis was performed on LightCycler480/IIi (ROCHE) with primers FLC Unspliced_LP and FLC Unspliced_RP for the unspliced sense FLC transcript and with primers FLC Spliced_LP and FLC Spliced_RP for the spliced sense FLC transcript. qPCR data was normalized to UBC (which was amplified with primers UBC-F and UBC-R). The primers are described in Table S1.

**Measuring COOLAIR Splicing Efficiency**

To measure the splicing efficiency of class II intron, 5 μg of total RNA isolated from seedlings were reverse-transcribed into cDNA, primed by Int1_RT, which is located in the exon 2 of class I and class II, (for locations of the primers, see also the illustration presented in Figure 3A). Resulting cDNA was used as template in qPCR reactions to amplify cDNA with the first small intron spliced by primers Int1_spliced_LP and Int1_spliced_RP, which covers the splicing junction. cDNA with the first small unspliced intron was amplified by primers Int1_unspliced_LP and Int1_unspliced_RP, which is located in the first small intron. Triplicates of all PCR reactions were performed and quantified against standard curves of cDNA dilutions. These data were then used to calculate the mean together with the spliced/unspliced ratio. RT− controls were always included to confirm absence of genomic DNA contamination.

To measure COOLAIR class II intron splicing efficiency, 5 μg of total RNA isolated from seedlings were reverse-transcribed into cDNA, primed by Class II unspliced F, and located in the last exon of all the class II antisense RNA. The resulting cDNA was used as template in qPCR to amplify spliced class II I with primers Class II-1_LP and Class II-1_RP, which cover the splicing junction; Class II intron 2 spliced with primers Class II-2_LP and Class II-2_RP, which cover the splicing junction; and FLC antisense big introns unspliced with primers Class II unspliced F and Class II unspliced R. Triplicate PCR reactions were performed and quantified against standard curves of cDNA dilutions before calculating the mean and spliced/unspliced ratio. RT− controls were always included to confirm absence of genomic DNA contamination.

**Measuring Polyadenylated COOLAIR**

The following primers were employed for the analysis of the COOLAIR transcripts: (a) for proximal poly(A) site transcript oligod(T) primer was used for the reverse transcription and forward primer, set1_RP, and reverse primer, LP_FLCl6polyA, used for the qPCR analysis (Liu et al., 2010), and (b) for the distal poly(A) site, oligod(T) primer was used for the reverse transcription and forward primer Set4_RP and reverse primer Set4_LP used for the qPCR analysis. qPCR reactions were performed in triplicates for each sample. Average values of the triplicates were normalized to the expression of total COOLAIR (which was amplified with Total COOLAIR_LP and Total COOLAIR_RP primers). The primers are summarized in the Table S1.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes seven figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2014.03.026.
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