FRIGIDA Delays Flowering in Arabidopsis via a Cotranscriptional Mechanism Involving Direct Interaction with the Nuclear Cap-Binding Complex[1][W]

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A major determinant of flowering time in natural Arabidopsis (*Arabidopsis thaliana*) variants is *FRIGIDA* (*FRI*). FRI up-regulates expression of the floral repressor *FLOWERING LOCUS C* (*FLC*), thereby conferring a vernalization requirement and a winter annual habit. FRI encodes a novel nuclear protein with no conserved domains except for two coiled-coil regions. A mutation in the large subunit of the nuclear cap-binding complex (CBC) suppresses FRI activity, so we have explored the connection between FRI and the nuclear CBC in order to gain further insight into FRI biochemical activity. Mutations in the small subunit of the CBC (*CBP20*) also suppress FRI up-regulation of *FLC*. CBP20 interacted directly with FRI in yeast and in planta, and this association of FRI with the 5′ cap was reinforced by an RNA ligase-mediated rapid amplification of cDNA ends assay that showed FRI decreased the proportion of *FLC* transcripts lacking a 5′ cap. Loss of CBP20 resulted in very low *FLC* mRNA levels and an increased proportion of unspliced *FLC* transcripts. FRI compensated for CBP20 loss, partially restoring *FLC* levels and normalizing the unspliced-spliced transcript ratio. Our data suggest that FRI up-regulates *FLC* expression through a cotranscriptional mechanism involving direct physical interaction with the nuclear CBC with concomitant effects on *FLC* transcription and splicing.

Many plant species overwinter before flowering in spring. In Arabidopsis (*Arabidopsis thaliana*), a major determinant of this life history strategy is FRIGIDA (FRI). FRI up-regulates the expression of *FLOWERING LOCUS C* (*FLC*), a gene encoding a MADS box transcriptional regulator that represses the expression of genes required to switch the shoot apical meristem from a vegetative to a reproductive fate (Michaels and Amasino, 1999; Sheldon et al., 1999). Prolonged cold down-regulates and epigenetically silences *FLC*, in a process known as vernalization, thus overcoming FRI effects and enabling flowering in spring. Vernalization has been shown to involve changes in histone modifications at *FLC*, providing an explanation for the mitotic stability of the vernalized state (Bastow et al., 2004; Sung and Amasino, 2004). FRI-mediated high-level expression of *FLC* has also been associated with changes in histone modifications, notably increased H3K4 trimethylation, a mark associated with actively expressed genes (He et al., 2004). FRI has also been shown to interact with SUPPRESSOR OF FRIGIDA4 (SUF4), which binds to the *FLC* promoter (Kim et al., 2006), suggesting that FRI might be involved in the transcriptional regulation of *FLC*.

Considerable allelic variation at FRI has been described and associated with flowering time variation in natural Arabidopsis accessions. Loss-of-function mutations have led to the independent evolution of rapid-cycling accessions (Johanson et al., 2000; Gazzani et al., 2003; Shindo et al., 2005), and the widely used rapid-cycling laboratory strains Columbia (Col) and Landsberg *erecta* both contain deletion alleles at *FRI* that account for their early flowering. It has been harder to interpret the functional consequences of the missense polymorphisms in FRI, and this has encouraged a more detailed analysis of FRI biochemical function. *FRI* encodes a novel protein with no domain that immediately suggests a function but two potential coiled-coil domains (Johanson et al., 2000) thought to interact with other proteins or nucleic acids. Much of our understanding has come from analysis of mutations that suppress FRI activity. Two of these encode *FRIGIDA-LIKE* (*FRL*) genes, *FRL1* and *FRL2*, with a low amino acid identity (approximately 28%) compared with *FRI* (Michaels et al., 2004; Schläppi, 2006). Other suppressors include mutations in FRIGIDA-ESSENTIAL1 and SUF4, proteins with zinc finger

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domains (Schmitz et al., 2005; Kim et al., 2006; Kim and Michaels, 2006), and *VERNALIZATION INDEPENDENCE/EARLY FLOWERING* genes, components of the Paf complex, required for chromatin remodeling and transcriptional elongation (Zhang et al., 2003; He et al., 2004), PHOTOPERIOD-INDEPENDENT EARLY FLOWERING1, an ATP-dependent chromatin-remodeling protein (Noh and Amasino, 2003), ACTIN-RELATED PROTEIN6, a putative component of a chromatin-remodeling complex (Choi et al., 2005; Deal et al., 2005; Martin-Trillo et al., 2006), EARLY IN SHORT DAYS4, a putative SUMO protease (Murtas et al., 2003), and ENHANCER OF AG-4 2 (HUA2), a protein involved in RNA processing (Doyle et al., 2005). The latter five also suppress the effects of mutations in the autonomous pathway (Zhang et al., 2003) and so are likely to define genes required for high-level *FLC* expression.

Mutation of RNA metabolism regulators, such as the large subunit of cap-binding protein complex (CBP80), also suppresses FRI up-regulation of *FLC* expression (Bezerra et al., 2004). The *cbp80* mutation was first identified as *abscisic acid hypersensitive1* (Hugouvieux et al., 2001) and has recently been shown to affect the mRNA metabolism of a number of Arabidopsis flowering time regulators (Kuhn et al., 2007, 2008). The nuclear cap-binding complex (CBC) is composed of two subunits, CBP80 (80 kD) and CBP20 (20 kD; Izaurralde et al., 1994), and is bound to the 5′ cap of mRNAs after a few nucleotides (20–30) have been synthesized by RNA polymerase II (Izaurralde et al., 1994). It has been suggested that the complex is involved in protecting the mRNA from degradation by exonucleases (Beelman and Parker, 1995) and that it interacts with the 3′ poly(A) machinery and facilitates nuclear export (Flaherty et al., 1997). It has also recently been shown to have dual roles in pre-mRNA splicing and microRNA (miRNA) processing in Arabidopsis (Laubinger et al., 2008). The nuclear CBC is replaced by elongation initiation factor 4 (eIF-4E) once the mRNA is exported to the cytoplasm (Gingras et al., 1999). The eIF-4E binds to the cap structure and to other translation initiation factors and enhances translation by promoting the interaction of the ribosomal subunits with the mRNA (Gingras et al., 1999).

mRNAs that lack a cap structure are efficiently degraded by exonucleases that also target aberrant mRNAs, for example, those containing premature termination codons, ensuring that truncated proteins are not translated (Parker and Song, 2004). In Arabidopsis, XRN1-like enzymes are present in multiple isoforms. XRN4 is the functional homolog of the yeast xrn1p (Souret et al., 2004) and can function in the cytoplasm (Kastenmayer and Green, 2000). In *xrn4*, the products of miRNA-mediated cleavage of *SCARECROW-LIKE* transcripts and other miRNA targets accumulate (Souret et al., 2004). Absence of a functional XRN4 also led to triggering of RNA silencing through the accumulation of an uncapped transcript of a transgene expressing an inducible shoot meristem regulator (STM:GR), linking mRNA-degradation pathways with RNA-silencing pathways (Gazzani et al., 2004). The uncapped transcripts provided the substrate for an RNA-dependent RNA polymerase to produce small interfering RNA, which ultimately silenced the transgene.

We have continued to explore the functional connection between FRI and the nuclear CBC and have investigated the loss of CBP20, physical interactions between FRI and the CBC components, and the effect of FRI on the 5′ cap of the *FLC* transcript. We have also investigated whether this activity is linked to RNA degradation pathways by exploring the effects of an *xrn4* mutation with and without FRI. The data suggest that FRI functions in independent pathways to XRN4 and is intimately involved in cotranscriptional processes linking the 5′ cap function, via a physical interaction of FRI and CBP20, with transcription and efficient splicing.

RESULTS

CBP20 Is Required for FRI Activity

Analysis of suppressors of *FRI* had revealed that the large subunit of the CBC, *CBP80*, was needed for FRI to up-regulate *FLC* transcript levels (Bezerra et al., 2004). Therefore, we investigated if the small subunit of the CBC, CBP20, was also required for the activity of FRI. The *cbp20* mutant has been previously described as conferring drought tolerance in Arabidopsis and having a serrated leaf phenotype (Papp et al., 2004). *cbp20* was crossed to a Col FRI line, and homozygous *FRI cbp20* individuals were identified. The Col FRI line had been generated by recurrent (nine generations) backcrossing of a Landsberg *erecta* line transformed with a genomic clone carrying an active *FRI* allele (called JU223 in Johanson et al., 2000) to Col. *FRI cbp20* flowered much earlier than *FRI CBP20* (Fig. 1A) but still showed the serrated leaf phenotype typical of *cbp20* (Supplemental Fig. S1), and this early flowering was associated with reduced *FLC* RNA levels (Fig. 1B). Therefore, the *cbp20* mutation does suppress FRI up-regulation of *FLC*. To ascertain how specific the *cbp20* suppression was to FRI function, we also analyzed the effect of *cbp20* on *fca-9*-dependent late flowering. *fca-9 cbp20* plants flowered much earlier than *fca-9* (Supplemental Fig. S2). Thus, *cbp20*, like *cbp80*, falls into the group of *FRI* suppressors that suppress both *FRI* and autonomous pathway functions.

FRI Physically Interacts with CBP20

As *cbp20* suppressed FRI function, we explored if there was a physical interaction between FRI and CBP20. A full-length CBP20 protein interacted with a full-length FRI protein in a yeast two-hybrid analysis (Supplemental Fig. S3) but did not interact with proteins required to mediate vernalization, for example, VRN1, VRN2, VRN5, and VIN3, or autonomous path-

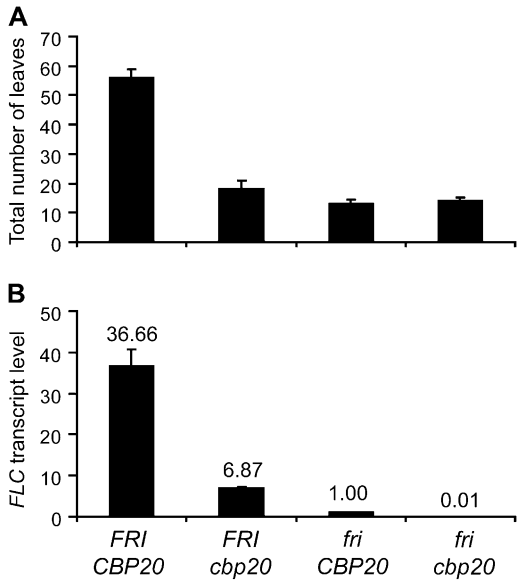

Figure 1. CBP20 is required for FRI-mediated up-regulation of *FLC*. A, Analysis of the flowering time of CBP20 and *cbp20*, with and without a functional *FRI* allele, was carried out (relative to Col-0; *fri CBP20*). Seeds of the different genotypes were sown on soil stratified for 3 d at 4°C and moved to the greenhouse at approximately 20°C and 16 h d. Ten plants were analyzed for each genotype, and means ± SE are shown. B, Q-RT-PCR analysis of *FLC* transcript levels in *cbp20*, with and without a functional *FRI* allele. RNA was extracted from seedlings grown for 7 d at 20°C under long days. *FLC* transcript levels were normalized to *TUB6* levels and are given relative to the Col-0 wild type without active *FRI* (*fri CBP20*). Values shown are means ± SE.

way components, for example FCA or FY (data not shown). The interaction with CBP20 was confirmed using coimmunoprecipitation assays following *Nicotiana benthamiana* transient expression experiments using FRI-FLAG- and CPP20-HA-tagged proteins (Fig. 2). Unrelated FLAG- and HA-tagged proteins did not interact under our assay conditions (S. Marquardt and C. Dean, unpublished data). Since mutations in the *FRI* homolog *FRL1* have also been found to suppress FRI up-regulation of *FLC* (Michaels et al., 2004), we also investigated the interaction of FRI and FRL1 in yeast. Previous studies had suggested that FRI and FRL1 did not interact in yeast (Kim et al., 2006; Kim and Michaels, 2006); however, we found that FRI interacted with FRL1 in two different yeast strains, albeit less strongly than CBP20 (Supplemental Fig. S3). To identify additional proteins that interact with FRI, a yeast two-hybrid screen was carried out and two interacting proteins, FRIGIDA-INTERACTING PROTEIN1 (FIP1; At2g06005) and FIP2 (At4g17060), were found, FIP1 interacting more weakly than FIP2 (Supplemental Fig. S3). There is one close homolog of FIP1 in the Arabidopsis genome (At5g20580), but otherwise FIP1 and FIP2 show no strong homology to each other or to other proteins in the current databases. A ClustalW analysis suggests that FIP2 shows very weak homology to the *Saccharomyces cerevisiae* NUP protein, a subunit of the nuclear pore complex. T-DNA insertions into the *FIP1* and *FIP2* genes were identified, and the loss-of-function alleles were crossed to Col *FRI*. The flowering time of plants homozygous for the T-DNA and *FRI* was found to be the same as that of the Col *FRI* parent (data not shown), so unlike *FRL1*, *CBP20*, and *CBP80*, loss of *FIP1* and *FIP2* is not sufficient to disrupt FRI up-regulation of *FLC*.

fri Mutations Increase Levels of Uncapped *FLC* RNA

Suppression of FRI up-regulation of *FLC* by components of the nuclear CBC and direct interaction of FRI and CBP20 led us to analyze whether FRI affects the proportion of *FLC* transcript that carries a 5′ cap. This was achieved using an assay based on 5′ RACE-PCR after ligation of an RNA adaptor (Gazzani et al., 2004). Only RNAs lacking a 5′ cap have the necessary phosphate group for the ligation step. RNA extracted from young seedlings of Col-0 and Col *FRI* was compared using *β-TUBULIN* as an internal control for RNA quantity and the levels of uncapped mRNA (Fig. 3). An additional control was made by removing the 5′ cap from all of the mRNAs in Col-0 and Col *FRI* before ligation with the RNA adaptor. The result shows that Col *FRI* has much higher levels of *FLC* than Col-0, with *β- TUBULIN* levels unaffected (Supplemental Fig. S4). Cloning and sequencing of the 683-bp major PCR fragment confirmed that it corresponded to the *FLC* transcript, with the sequence starting in the 5′ RNA adapter and ending at the 3′ end of the *FLC*-specific primer. The absence of *FRI* led to an increase (approximately 4-fold) in the specific *FLC* fragment in Col-0 (*fri*), supporting the conclusion that *FRI* increases the proportion of *FLC* transcript that contains a 5′cap (Fig. 3). This effect appears to be specific to FRI and not just due to high levels of *FLC* RNA. *fca-9* and *fld-4* seedlings, which have levels of *FLC* comparable to FRI+ plants (Bäurle and Dean, 2006), showed levels of uncapped *FLC* RNA equivalent to Col-0 (Fig. 3C). Col *FRI* and Col-0 plants were also vernalized and the assay repeated. Vernalization did not appear to antag-

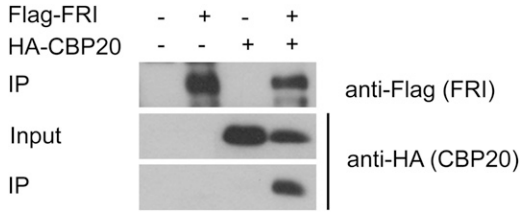

Figure 2. FRI physically interacts with CBP20 after transient expression in *N. benthamiana*. N-terminally tagged fusion proteins (Flag-FRI and HA-CBP20) were expressed in *N. benthamiana* leaves. Protein extracts were immunoprecipitated (IP) with anti-Flag M2 agarose, and after washing, the eluate was analyzed on western blots with anti-Flag and anti-HA serum, respectively. The input lane was loaded with 10% of the raw protein extract.

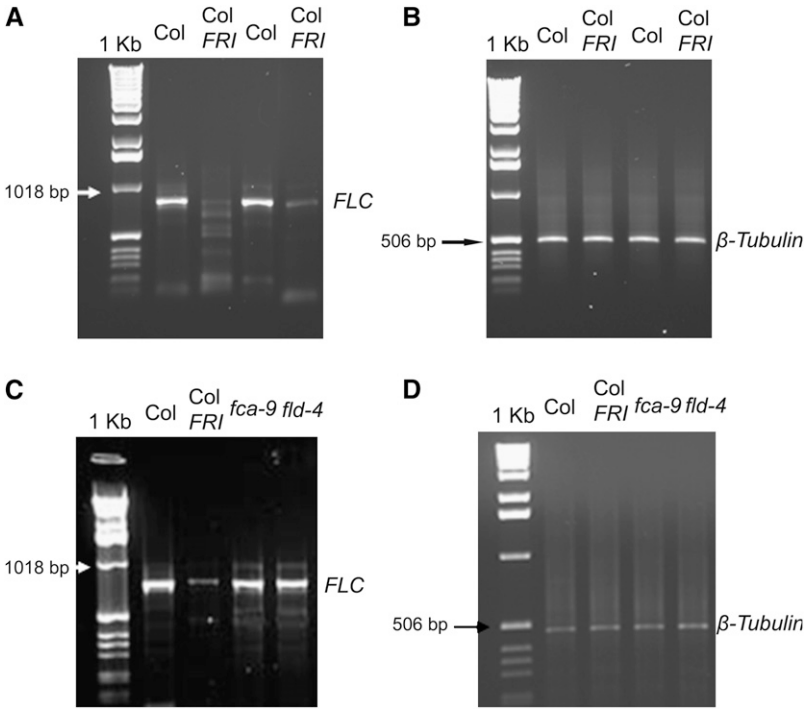

Figure 3. FRI increases the proportion of *FLC* RNA with a 5′ cap. Poly(A) RNA extracted from 7-d-old Col-0 and Col *FRI* seedlings was ligated to an RNA adapter. The product was amplified with primers specific for the RNA adapter and either *FLC* (A) or *β-TUBULIN* (B). Two independent Col FRI and Col-0 samples are shown. Poly(A) RNA extracted from 7-d-old Col-0, Col *FRI*, *fca-9*, and *fld-4* was similarly ligated to an RNA adaptor and amplified with primers specific for the RNA adapter and either *FLC* (C) or *β-TUBULIN* (D). Relative abundance was assessed through densitometric analysis of the gel fragments.

onize FRI action by affecting levels of uncapped *FLC* mRNA (Supplemental Fig. S5).

We next analyzed the effect of mutations in the FRI interactors on *FLC* capping using the same ligation-mediated PCR assay. Consistent with their suppression of FRI up-regulation of *FLC*, *cbp20* and *frl1* mutations suppressed the FRI reduction in *FLC* RNA lacking a 5′ cap (Fig. 4; Supplemental Fig. S6). *fip1* and *fip2*, in contrast, did not (Supplemental Fig. 6), reinforcing the correlation between an influence on the 5′ *FLC* cap and an ability to suppress FRI function.

xrn4 Shows an Additive Interaction with *FRI*, Indicating That They Function in Independent Pathways to Regulate *FLC*

The presence of a 5′ cap structure has previously been shown to protect RNA from degradation by 5′-3′ exonucleases (Parker and Song, 2004), such as the cytoplasmically localized *XRN4* in Arabidopsis (Souret et al., 2004). To investigate whether the influence of FRI on the *FLC* 5′ cap was through changed RNA stability, we analyzed *FLC* levels in *fri* and *FRI* plants carrying *xrn4-1*. *xrn4-1* increased *FLC* levels in both *fri* (Landsberg *erecta*) seedlings and FRI+ genotypes, interacting additively with FRI (Fig. 5). This was also true when *fca*-9 was combined with *xrn4*. Thus, it seems that FRI and FCA function in genetically independent pathways to XRN4 to regulate *FLC*. *xrn4* increased *FLC* levels, suggesting that there is a significant proportion of XRN4-accessible *FLC* message in the cytoplasm that is degraded by XRN4 activity.

FRI Suppresses *cbp20*-Induced Increases in the Ratio of Unspliced to Spliced *FLC* Transcript

The function of the nuclear CBC is intimately connected with messenger ribonucleoprotein assembly and transcript splicing in many organisms (Proudfoot et al., 2002). CBP20 and CBP80 have also recently been shown to have dual roles in splicing of many tran-

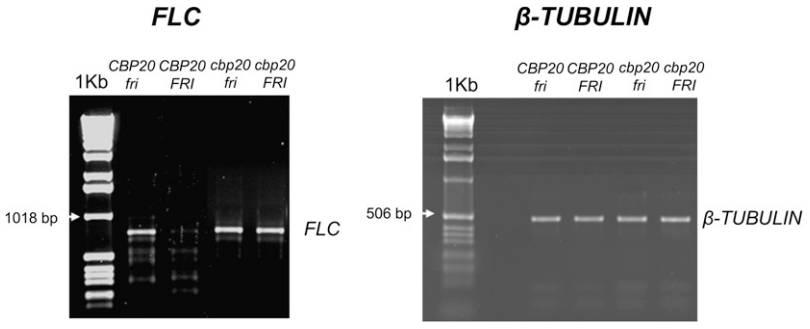

Figure 4. Analysis of *FLC* transcripts lacking a 5′ cap structure in the *cbp20* mutant. The mRNA extracted from Col-0, Col *FRI*, *cbp20*, and *cbp20 FRI* was ligated to an RNA adapter. The mRNA was then used for cDNA synthesis and *FLC* amplification by PCR. Using the same cDNA samples, *β-TUBULIN* was amplified as an internal control.

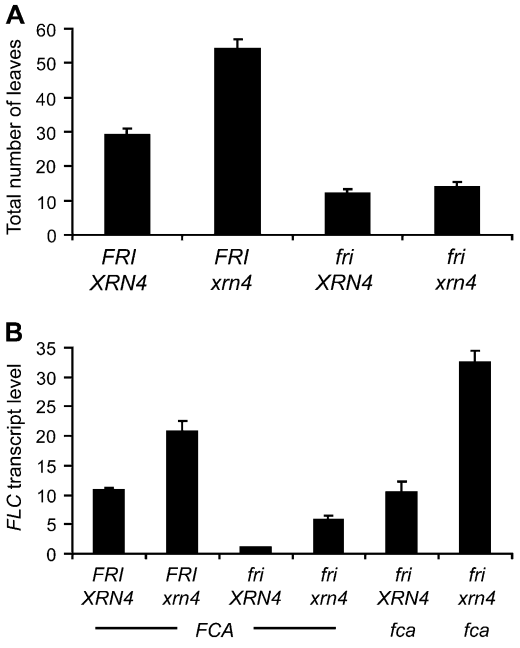

Figure 5. XRN4 and FRI function independently to regulate *FLC*. A, Analysis of the flowering time of *xrn4* (Landsberg *erecta* background), with and without a functional *FRI* allele. Seeds from the different genotypes were sown on soil and grown in the greenhouse at approximately 20°C and long-day conditions. Ten plants were analyzed for each genotype, and means ± SE are shown. B, Q-RT-PCR analysis of *FLC* transcript levels in the *xrn4* mutant, with and without a functional *FRI* allele and with and without a functional *FCA* allele. RNA was extracted from seedlings grown for 7 d at approximately 20°C under long days. *FLC* transcript levels were normalized to *TUB6* levels and are given relative to the Landsberg *erecta* wild type without active *FRI* (*fri XRN4*). Values shown are means ± SE.

scripts and miRNA processing in Arabidopsis, with many partially spliced transcripts being found in CBC mutants, such as *cbp20* (Laubinger et al., 2008). In general, a single intron from each gene was retained in *cbp20*, with the splicing of the first introns appearing to be most sensitive to loss of CBC. Therefore, we developed a quantitative reverse transcription (Q-RT)-PCR assay to compare both spliced and unspliced *FLC* transcripts in wild-type and different mutant genotypes. The splicing of intron 3 was chosen to represent "spliced" *FLC* transcript, but we found similar data if intron 6 was assayed (data not shown). FRI clearly upregulated both unspliced and spliced *FLC* transcripts, consistent with an effect on transcription. The *cbp20* mutation had a much higher proportion, approximately 10-fold more, of unspliced to spliced *FLC* transcripts (approximately 22% in *cbp20* compared with 2% in FRI and CBP20 plants; Fig. 6) but very low levels of *FLC* RNA. FRI, however, can somewhat compensate for CBP20 loss, partially restoring *FLC* levels and normalizing the unspliced-spliced *FLC* transcript ratio. FRI perhaps functions as part of an extended CBC, where the components can partially function for each other but where the intact complex is required for maximal integration of transcription and splicing.

DISCUSSION

A requirement for vernalization influences whether a plant overwinters before flowering, thus flowering only once a year. In Arabidopsis, a major determinant of vernalization requirement is FRI, through its upregulation of the strong floral repressor *FLC*. FRI protein is a positively charged, Lys-rich protein with two strongly predicted coiled-coil domains but no other clear homology to other proteins or domains of known function. FRI results in increased H3K4 trimethylation at *FLC* and interacts with SUF4, which directly associates with *FLC* chromatin, so FRI could act as a transcriptional activator. In the study described here, we have pursued

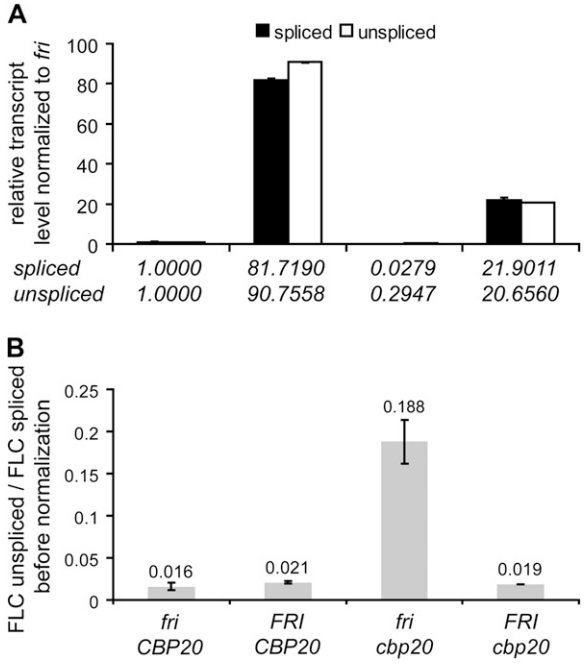

Figure 6. FRI suppresses the *cbp20*-induced increase in the ratio of unspliced to spliced *FLC* transcript. A, Levels of spliced and unspliced *FLC* transcripts were determined by Q-RT-PCR analysis in different backgrounds and were normalized to *TUB6* and separately to the spliced and unspliced levels, respectively, of the *fri CBP20* (Col-0) background (relative *FLC* transcript level in genotype g = *FLC* transcript level in genotype g/*FLC* transcript level in *fri CBP20* with *FLC* transcript level in genotype g = $2^{(Ct\ (TUBg)\ -\ Ct\ (FLCg))}$). Raw Ct (threshold cycle) values are given in Supplemental Table S1. B, The ratio of *FLC* unspliced transcript levels to *FLC* spliced transcript levels, averaged over two independent biological replicates, showed that the level of spliced *FLC* transcripts is around 50-fold that of the unspliced *FLC* transcripts (equal amplification efficiency of both amplicons was confirmed in standard curves) in *CBP20 fri* (Col-0), *CBP20 FRI*, and *cbp20 FRI* but around 5-fold that of *cbp20 fri* unspliced transcripts. The ratio between unspliced and spliced *FLC* transcripts was estimated as ratio = $2^{(Ct\ (spliced)\ -\ Ct\ (unspliced))}$. Values shown are means ± SE.

the observation that FRI up-regulation of *FLC* is suppressed by a mutation in the large subunit of the nuclear CBC, CBP80 (Bezerra et al., 2004). Our data suggest that FRI up-regulates *FLC* expression through a cotranscriptional mechanism linking 5′ cap function, through a direct physical interaction with the nuclear CBC, with increased transcription and efficient splicing.

The molecular machinery regulating cotranscriptional RNA metabolism is still not well understood, particularly in plants. Work in yeast and humans has shown that the CBC, composed of the two conserved subunits CBC80 and CBC20, associates with the CTD of RNA polymerase II and is required for many aspects of RNA splicing, RNA stability, and export and has been proposed to affect transcriptional elongation via a checkpoint mechanism (Gamberi et al., 1997; Shatkin and Manley, 2000). Our analysis reveals that FRI is a direct interactor of the CBC, with suppression of FRI activity by both *cbp20* and *cbp80* providing in vivo endorsement of the importance of the CBC for *FRI* function.

FRI function is also suppressed by mutations in the *FRI* homologs *FRL1* and *FRL2*. FRL1 appears to weakly interact in yeast with FRI, and we have preliminary data that FRL2 interacts with FRL1 (data not shown). These FRI-related proteins, therefore, may all form a complex in vivo. The additional FRI interactors identified in the yeast two-hybrid screens encode novel proteins with no clearly identifiable functional domains, so these do not help to define the biochemical function of any putative complex. Mutations in these genes do not impair *FLC* regulation, indicating that either *FLC* is not a target of those proteins or there is genetic redundancy, the latter being feasible due to the very close homolog of *FIP1* in the Arabidopsis genome. *frl1* specifically suppresses FRI function, whereas *cbp20* suppresses both FRI and *fca* mutations. Thus, these FRI interactors both influence *FLC* capping but fall into different groups of FRI suppressors: those that are specific for FRI and those that suppress *FLC* up-regulation more generally. FRI suppressors with apparently different specificities might appear to influence a common mechanism through intimate connection of the cotranscriptional processes linking 5′ capping, 3′ end formation, nuclear export, and transcriptional elongation. Disruption of one process might feed back and affect the others. Further analysis of the degree of capping, transcription termination, export efficiency, H3K4 methylation, and H3K36 methylation in all of the FRI suppressors will be an important next step.

Loss of CBP80 and thus CBC function has been shown to disrupt RNA metabolism of a number of flowering time regulators (Kuhn et al., 2007, 2008). Inclusion of intron 1 in *FLC* transcripts, premature polyadenylation in *FLM*, and cis-natural antisense transcripts of *CO* were all found to occur at higher levels in *cbp80*. However, the presence of these RNA transcripts also in wild-type plants suggested that the loss of CBC quantitatively rather than qualitatively affects the RNA metabolism of these genes. In contrast, we have found that *cbc20* results in very low *FLC* transcript levels and a change in the ratio of unspliced to spliced *FLC* transcript. This appeared to be suppressed by FRI activity, which led to increased *FLC* transcription, restoration of the unspliced-spliced ratio, and a reduced level of uncapped *FLC* transcript. Reduced levels of uncapped *FLC* RNA do not account for the FRI-mediated increase in *FLC* expression as judged through the analysis with the *xrn4* mutant. *xrn4* and FRI appeared to increase *FLC* RNA through additive and therefore independent mechanisms. Taken together, FRI appears to influence cotranscriptional processes linking 5′ cap function with transcription and efficient splicing. It will be interesting to investigate the effects of FRI on miRNA processing and on the set of endogenous Arabidopsis transcripts that accumulate in *xrn4* (also known as *ein5*; Gregory et al., 2008; Laubinger et al., 2008).

Proteins that interact with the CBC have recently been shown to include components of the conserved mRNA export machinery in humans (Cheng et al., 2006). The human export machinery (TREX) contains the exon-junction complex proteins UAP56 and Aly (Sub2p and Yra1 in yeast) as well as the multisubunit THO complex (Jimeno et al., 2002; Strässer et al., 2002; Masuda et al., 2005). In yeast, Sub2p and Yra1 are cotranscriptionally recruited to nascent transcripts by the THO complex. In humans, TREX is recruited in a splicing- and cap-dependent manner to the 5′ end of the mRNA (Cheng et al., 2006). Loss of TREX also leads to reduced transcription, possibly through nascent transcripts that are not efficiently exported interfering with the transcribing polymerase. The functional outcome is a link between the 5′ end of the RNA with transcription elongation and efficient nuclear export (Cheng et al., 2006). A functional equivalence between the FRI protein complex(es) and the nuclear export machinery is a particularly attractive model given the need for a conserved nuclear pore protein to maintain high *FLC* expression levels (Jacob et al., 2007). The low homology of functionally similar yeast and mammalian nuclear export proteins makes it difficult to identify clear homologs of some of the TREX complex in the Arabidopsis genome. Further biochemical and genetic analyses of FRI effects on these processes are required to fully understand how far the parallels extend from yeast to plants. This should not only improve our knowledge of cotranscriptional regulation in plants, but a mechanistic understanding of FRI function will allow functional dissection of the considerable allelic variation at FRI and its impact on the evolution of different life history strategies.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

For Arabidopsis (*Arabidopsis thaliana*) plants grown in soil, seeds were sown in plastic pots. Seeds were vernalized for 4 or 6 weeks immediately after

sowing at 5°C, with an 8-h photoperiod (photosynthetically active radiation, 9.5 μmol m^{-2} s^{-1}; red-far red ratio, 3.9). Nonvernalized plants were stratified for 2 d in the same conditions. Plants were subsequently moved from the cold to extended short days at 20°C in a controlled-environment room (Sanyo Gallenkamp) with a 16-h photoperiod (photosynthetically active radiation, 114 μmol m^{-2} s^{-1}) composed of 10 h from 400-W Wotan metal halide lamps and 100-W tungsten halide lamps (red-far red ratio, 2.4) and a 6-h extension of exclusively tungsten halide lamps (red-far red ratio, 0.66). Young seedlings were transferred to trays with 40 cells of 2 × 2 cm. Flowering time was measured by counting total leaf number, which was scored as the number of rosette leaves plus cauline leaves. Plants grown on plates were sown aseptically in petri dishes containing GM medium. The *cbp20* and *xrn4* mutants were kind gifts from Dr. István Papp (Agricultural Biological Center, Pest, Hungary) and Robert Sablowski (John Innes Centre, Norwich, UK), respectively.

Gene Expression Analysis

RNA was isolated using a scaled-down protocol described by Etheridge et al. (1999). For real-time Q-RT-PCR, total RNA was treated with TURBO DNA-free (Ambion), and 2 μg was reverse transcribed with SuperScript III (Invitrogen) according to the manufacturer's instructions. cDNA was used at 0.25 μL per Q-PCR run in a 20-μL reaction volume using SYBR Green JumpStart Taq ReadyMix (Sigma) and an MJ Research Opticon2 instrument. Expression was normalized to *β-TUBULIN6* using the comparative C_T (threshold cycle) method. Primer sequences were as follows: 5′-AGCCAAGAAGACCGAACTCA-3′ and 5′-TTTGTCCAGCAGGTGACATC-3′ for spliced *FLC*, 5′-CGCAATTTTCATAGCCCTTG-3′ and 5′-CTTTGTAATCAAAGGTGGAGAGC-3′ for unspliced *FLC*, and 5′-GTCATCTGCAGTTGCGTCTT-3′ and 5′-GGTGAAGGAATGGACGAGAT-3′ for *TUB6*.

Coimmunoprecipitation

For transient overexpression of Flag-FRI and HA-CBP20 in *Nicotiana benthamiana* leaves, the Flag tag and HA tag, respectively, were fused N terminally to the FRI and CBP20 cDNA and cloned into pBIN61 (Bendahmane et al., 2002). Expression in *N. benthamiana* leaves and coimmunoprecipitation were performed as described (Greb et al., 2007). Briefly, for coimmunoprecipitation, protein extracts were incubated with anti-Flag M2 agarose (Sigma; A2220). After washing, bound proteins were eluted and analyzed by western blotting.

RNA Ligase-Mediated RACE Assay

Detection of RNA 5′ ends was performed using the GeneRacer Kit (Invitrogen), without the initial decapping reaction, a protocol adapted from Llave et al. (2002) and Gazzani et al. (2004). After isolation of poly(A) RNA (Oligotex; Qiagen), the GeneRacer RNA oligonucleotide was ligated to exposed 5′ ends and RT reaction was carried out using an oligo(dT) primer. A total of 250 ng of poly(A) RNA was ligated to the RNA adaptor using 5 units of T4 RNA ligase at 37°C for 1 h, follow by removal of proteins from solution with phenol/chloroform extraction and ethanol precipitation. The RNA obtained was used for cDNA synthesis with 200 units of SuperScript III reverse transcriptase enzyme and used for PCR amplification using a Taq polymerase enzyme (Qiagen). A 10-cycle hot-start PCR was performed (94°C for 4 min followed by 10 cycles of 94°C for 30 s, 68°C for 30 s, and 72°C for 90 s) using a primer specific for the GeneRacer RNA oligonucleotide (5′-CGACTGGAGCACGAGGACACTGA-3′) and an *FLC*-specific primer (5′-TGGGAGAGTCACCGGAAGATTGTCG-3′). The PCR products were purified with the QIAQuick PCR purification kit (Qiagen) and used as templates for a second 30-cycle hot-start PCR (94°C for 4 min and then 30 cycles of 94°C for 30 s, 68°C for 30 s, and 72°C for 90 s) with GeneRacer 5′ nested primer (5′-ACATGGACTGAAGGAGTAGAAACGAG-3′) and *FLC* nested primer (5′-TCCATCTCAGCTTCTGCTCCCACATGA-3′) to decrease the nonspecific amplification. These PCR products were separated on a 1% agarose gel and transferred to a nylon filter (Hybond N^+; Amersham) by Southern blotting. The cloning and sequencing of the most prominent PCR product (approximately 1 kb) confirmed that it corresponded to full-length *FLC* cDNA. Similar results (amplification of the full length *FLC* cDNA, with no detectable cleavage products) were obtained using the GeneRacer 5′ primer and a primer directed to the 3′ end of *FLC*.

Yeast Two-Hybrid Analysis

A nonamplified yeast library was generated using cDNA made from RNA extracted from Col *FRI* plants and cloned in pGBT9 (Stratagene). This was transformed into the yeast strain YRG2, and transformants were plated in the absence of Trp, Leu, and His (selective minimal medium) and incubated at 28°C for 6 d. The medium was supplemented with 20 mM 3-aminotriazole to reduce false positives from the leaky His promoter. Interactions were also analyzed in yeast strain PJ69. After 4 d of incubation at 28°C, yeast cells were observed on the selection plates containing synthetic dextrose medium lacking Leu, Trp, adenine, and His and grown until colonies had formed.

Sequence data from this article can be found in the GenBank data libraries under accession numbers NM_126614 (FIP1) and NM_117810 (FIP2).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. *cbp20* FRI shows leaf serrations.

Supplemental Figure S2. *cbp20* can suppress *fca* late-flowering phenotype.

Supplemental Figure S3. Yeast two-hybrid interaction analysis reveals an interaction between FRI and FRL1 and two novel FRI interactors, FIP1 and FIP2.

Supplemental Figure S4. *FLC* levels in Col-0 and Col *FRI* after the removal of the 5′ cap structure.

Supplemental Figure S5. Vernalization effect on *FLC* transcripts lacking a 5′ cap structure in Col-0 and Col *FRI*.

Supplemental Figure S6. Effect of FRI interactors on the level of uncapped *FLC* RNA.

Supplemental Table S1. Ct (threshold cycle) values from the *FLC* and *β-TUBULIN* Q-PCR analysis.

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