Autonomous Pathway: FLOWERING LOCUS C Repression through an Antisense-Mediated Chromatin-Silencing Mechanism

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The timing of flowering is vital for plant reproductive success and is therefore tightly regulated by endogenous and exogenous cues. In summer annual Arabidopsis (Arabidopsis thaliana) accessions, like Columbia-0, rapid flowering is promoted by repression of the floral repressor FLOWERING LOCUS C (FLC). This is through the activity of the autonomous pathway, a group of proteins with diverse functions including RNA 3′-end processing factors, spliceosome components, a transcription elongation factor, and chromatin modifiers. These factors function at the FLC locus linking alternative processing of an antisense long noncoding RNA, called COOLAIR, with delivery of a repressive chromatin environment that affects the transcriptional output. The transcriptional output feeds back to influence the chromatin environment, reinforcing and stabilizing that state. This review summarizes our current knowledge of the autonomous pathway and compares it with similar cotranscriptional mechanisms in other organisms.

A BRIEF OF HISTORY OF THE AUTONOMOUS PATHWAY

The importance of flowering in agriculture and the ease of characterizing flowering phenotypes make flowering time one of the most extensively studied plant traits. The earliest Arabidopsis (Arabidopsis thaliana) flowering mutant identified was luminidependens (ld; Rédei, 1962), a mutant later classified into the autonomous pathway (Lee et al., 1994). Systematic screening and characterization of mutants affecting flowering time identified a series of mutants that were classified into different groups based on their flowering behavior (Koornneef et al., 1991, 1998a, 1998b; Alonso-Blanco et al., 1998). Among these, one group of mutants showed delayed flowering in both long days and short days, and their flowering was accelerated by prolonged cold and low red to far-red light conditions. These were grouped into the autonomous pathway (autonomous of photoperiod).

Martin Koornneef and others in the 1990s and 2000s identified fpa, fca, fy, fve, flk, ld, and fld as mutants of the autonomous pathway (Lee et al., 1994; Sanda and Amasino, 1996; Koornneef et al., 1998a, 1998b; Lim et al., 2004). Cloning of these genes revealed that FPA (Schomburg et al., 2001), FCA (Macknight et al., 1997), and FLK (Lim et al., 2004) are all RNA-binding proteins. FY is a homolog of the budding yeast (Saccharomyces cerevisiae) RNA 3′-end processing factor Polyadenylation factor 1 subunit 2 (Pfs2p; Simpson et al., 2003), and...
LD is a homeodomain-containing protein (Lee et al., 1994; Aukerman et al., 1999). FLOWERING LOCUS D (FLD) is a histone Lys-4 demethylase (He et al., 2003; Liu et al., 2007), and FVE is a homolog of human (*Homo sapiens*) retinoblastoma-associated protein, often found in histone deacetylase complexes (Ausín et al., 2004). Genetic interactions between these mutants are nonlinear and complex (Koornneef et al., 1998a, 1998b). For example, in the Landsberg background, the *fps fyl* double mutant is lethal, indicating that FPA and FY have redundant and essential functions in addition to flowering time control, but in Columbia that interaction is nonlethal (Koornneef et al., 1998a, 1998b). FCA and FPA both act (at least partly) through the histone demethylase *FLD*, while *FVE* acts independently of *FCA* but may have a more complex interaction with FPA (Baurle and Dean, 2008).

Despite the complex genetic interactions among these proteins, their activities in flowering all converge on the regulation of a single gene, *FLOWERING LOCUS C (FLC)*, which encodes a MADS box protein that functions as a central repressor of flowering time in Arabidopsis (Michaels and Amasino, 1999, 2001; Sheldon et al., 1999). To date, FCA-mediated repression of *FLC* is perhaps the best-understood part of the autonomous pathway components. FCA is a nuclear RNA-binding protein that functions in regulating alternative RNA 3'-end processing. FCA interacts with FY though a WW motif (in FCA)-PPLP (in FY) interaction (Simpson et al., 2003; Henderson and Dean, 2004). Both proteins are required for negative autoregulation of FCA itself, through promoting the usage of a proximal polyadenylation site within *FCA* intron 3, therefore inhibiting the production of the functional isoform (Macknight et al., 2002; Quesada et al., 2003; Simpson et al., 2003). FCA and FPA function is not limited to flowering time gene regulation; they have been shown to promote proximal polyadenylation of a large number of Arabidopsis transcripts genome wide (Sonmez et al., 2011; Duc et al., 2013).

The mechanism of how the autonomous pathway represses *FLC* was largely uncovered by identification of the components involved through genetic screening of suppressors of overexpressed FCA (sof; Fig. 1A). In the progenitor line, expression of *FLC* is monitored by an *FLC-LUC* transgene made by translational fusion of firefly luciferase coding sequence into exon 6 of whole *FLC* genomic DNA sequence. A second transgene overexpressing only the spliced functional form of FCA mRNA (35S:FCA<sub>y</sub>) makes the line early flowering with very low levels of *FLC*. The line was further sensitized by introducing an active *FRI* allele, which would lead to up-regulated *FLC* when FCA-mediated repression is attenuated. Therefore, mutations disrupting the function of FCA, even only partially, were recovered by mutagenesis after screening for late-flowering mutants with an increased *FLC-LUC* expression.

Extensive sof mutant screening recovered several alleles of *fps* and *fyl*, indicating that FCA requires FPA and FY to suppress *FLC* (Liu et al., 2010). Other factors identified as sof mutants include CstF64 and CstF77, which are essential factors important for 3' processing and polyadenylation (Liu et al., 2010); FLD, a homolog of LSD1 H3K4me2 demethylase (Liu et al., 2007); PRP8, a core component of the spliceosome (Marquardt et al., 2014); CDKC2, a homolog of Positive transcription elongation factor b (P-TEFb; Wang et al., 2014); and FL2L2, a structural protein that promotes FCA liquid-liquid phase separation (LLPS; Fang et al., 2019).

In addition to the factors identified through sof mutant screens, several other autonomous pathway components have been described over the last few years. SR45, a splicing factor, is required for *FLC* repression (Ali et al., 2007). PCSF4 (Xing et al., 2008) and HLP1 (Zhang et al., 2015), part of the RNA 3'end processing machinery that regulates alternative polyadenylation of FCA transcripts, both repress *FLC*. PRMT5, an Arg methyltransferase, represses *FLC* by influencing splicing of *FLK* pre-mRNA (Deng et al., 2010). TAF15b (TATA-binding protein-associated factor), a member of the transcription factor IID complex, represses *FLC* and binds at the transcription start sites of both *FLC* and COOLAIR (Eom et al., 2018). In addition, two Gly-rich RNA-binding proteins, GRP7 and GRP8,
fine-tune FLC expression within the autonomous pathway (Steffen et al., 2019). In the following section, we discuss our current understanding of how the autonomous pathway works with a focus on FCA-mediated repression of FLC.

THE AUTONOMOUS PATHWAY: COTRANSCRIPTIONAL PROCESSING LINKED TO CHROMATIN SILENCING

Identification of fpa, fy, cstf64, and cstf77 as FCA suppressors revealed a link between RNA 3′-end processing and FCA-mediated repression of FLC (Liu et al., 2010). This became clearer when it was discovered that RNA pol II C-terminal domain as well as other elongation factors (Marshall and Price, 1995; Fujinaga et al., 2004; Yamada et al., 2006). Interestingly, the cdkc;2 mutation has the opposite effect on endogenous FCOAIR transcription. Therefore, proximal splicing and polyadenylation of FCOAIR is important for FCA-mediated FLC repression (Fig. 1B).

One intriguing question is whether FCOAIR transcripts, or their transcription, are more important for this repression. This was partially revealed by the discovery of another sof mutant, cdkc;2 (Wang et al., 2014). Evidence from mammals to Arabidopsis indicates that CDKC;2 is critical for efficient transcriptional elongation, particularly during the transition from initiation to productive elongation (Fujinaga et al., 2004; Fülöp et al., 2005; Cui et al., 2007; Ni et al., 2008; Antosz et al., 2017). P-TEFb is a kinase that promotes phosphorylation of Ser-2 of the RNA polymerase II (Pol II) C-terminal domain as well as other elongation factors (Marshall and Price, 1995; Fujinaga et al., 2004; Yamada et al., 2006). Interestingly, the cdkc;2 mutation has the opposite effect on endogenous FCOAIR at the FLC locus and a FCOAIR:LUC transgene (Wang et al., 2014). Comparison of the two revealed the feedback mechanisms between transcriptional output and chromatin environment. The cdkc;2 mutation decreased FCOAIR:LUC expression (a transgene containing the FCOAIR promoter driving expression of its own exon 1-intron 1 fused with the LUC gene) but lacking the rest of the FLC gene; Sun et al., 2013), consistent with the function of CDKC;2 as a positive elongation factor (Wang et al., 2014). However, at the endogenous FLC locus, cdkc;2 increased the absolute level of FCOAIR, although the proportion of proximal polyadenylated FCOAIR decreased (Wang et al., 2014). These results reveal that the process of polyadenylation at the proximal FCOAIR site is required to deliver a chromatin environment that suppresses expression of the whole locus. When this mechanism is perturbed and the silencing chromatin environment is not established, transcriptional repression is released, with both sense and antisense transcription increased. They further indicate that the absolute amount of proximal FCOAIR by itself is unlikely to be key to this repression mechanism. Therefore, although the detailed mechanism and feedbacks involved are still unclear, it is likely that a series of cotranscriptional events governed by PRP8, CDKC;2, and termination-related factors, rather than the transcripts themselves, are important for FCA-mediated repression of FLC (Fig. 1B).

The most unexpected sof mutant obtained so far is perhaps fld (Liu et al., 2007), a mutation of a homolog of human LSD1, which encodes a histone demethylase that removes H3K4me2. FLD associates with FLC chromatin and is responsible for demethylylation of H3K4me2 mainly in the body of the FLC gene (Liu et al., 2007; Wu et al., 2016). This occurs in parallel with a decrease in H3K36me3 and an increase in H3K27me3 at FLC chromatin (Lopez-Vernaza et al., 2012; Wu et al., 2016). The fld mutation genetically suppresses the function of both FCA and FPA (Liu et al., 2007; Bäurle and Dean, 2008), indicating that it functions downstream of FCOAIR proximal polyadenylation in the chromatin-silencing mechanism at FLC.

Currently, how exactly FCA- and FPA-mediated cotranscriptional RNA processing events are linked to the activity of FLD is unknown. However, as discussed in the following section, evidence from other systems indicates that RNA-binding proteins are frequently involved in cotranscriptional events and chromatin-based repression mechanisms.

PARALLEL EXAMPLES OF COTRANSCRIPTIONAL PROCESSING LINKED WITH CHROMATIN GENE REPRESSION

Perhaps the best parallel mechanism to FCA-mediated FLC repression is RNA interference (RNAi)-independent heterochromatin gene silencing in fission yeast (Schizosaccharomyces pombe). In S. pombe, heterochromatin is maintained through two major pathways. The first pathway involves an RNA-induced transcriptional silencing complex, which recruits H3K9 methyltransferase (Clr4; Reyes-Turcu and Grewal, 2012; Martienssen and Moazed, 2015). The second RNAi-independent pathway includes the remodeler complex SHREC (Sugiyama et al., 2007), RNA exonuclease Dhp1/Rat1/Xrn2 (Chalamcharla et al., 2015; Tucker et al., 2016), the termination-related RNA-binding protein Seb1 (Marina et al., 2013), and RNA quality control factors Mlo3/Yra1 and Rpr6 (Reyes-Turcu et al., 2011). Both pathways depend on H3K9me to maintain silencing (Reyes-Turcu et al., 2012; Wu et al., 2016). The ratio of proximal splicing and polyadenylation in FCA-mediated repression was further demonstrated through examination of the sof mutants prp8 and cdkc;2. PRP8 is a positive and essential splicing factor. At COOLAIR, proximal polyadenylation site usage is associated with splicing of a short intron, a process dependent on PRP8 (Marquardt et al., 2014; Fig. 1B). Mutation of the 3′ splice site of this short intron prevented FLC up-regulation in the prp8 background and shifted the polyadenylation site usage toward the distal site (Marquardt et al., 2014). Therefore, proximal splicing and polyadenylation of COOLAIR is important for FCA-mediated FLC repression (Fig. 1B).

Evidence from mammals to Arabidopsis indicates that RNA-binding proteins are frequently involved in cotranscriptional events and chromatin-based repression mechanisms.
and Grewal, 2012; Martienssen and Moazed, 2015). Although the histone marks involved are different, there are interesting similarities between FCA-mediated FLC repression and Seb1-mediated heterochromatin silencing.

Seb1 is a homolog of Nrd1 (Mitsuzawa et al., 2003), part of the Nrd1-Nab3-Sen1 complex in S. cerevisiae that promotes transcriptional termination and decay of a class of unstable noncoding RNAs called cryptic unstable transcripts (Steinmetz et al., 2001; Arigo et al., 2006; Thiebaut et al., 2006; Vasiljeva and Buratowski, 2006). Loss of Seb1 in S. pombe leads to release of heterochromatin gene silencing accompanied by partial loss of H3K9me, but without alteration of the RNAi pathway (Marina et al., 2013). Seb1 and the RNAi-dependent pathway function redundantly to a certain extent in H3K9me-mediated silencing, as this mark is completely lost only when both pathways are inactivated (Marina et al., 2013). RNA immunoprecipitation-quantitative PCR experiments showed that Seb1 binds to RNAs derived from centromeric repeats (Marina et al., 2013). Such noncoding RNAs are mainly transcribed during the S-phase of the cell cycle and are important for RNAi-dependent and -independent silencing (Djupedal et al., 2005; Kato et al., 2005; Chen et al., 2008). Therefore, Seb1 seems to play a direct role in this silencing mechanism, involving its binding of RNA (Fig. 2A).

Related to Seb1, a recent study identified components of the cleavage and polyadenylation factor (CPF) complex as positive regulators of RNAi-independent heterochromatin silencing (Vo et al., 2019). A YTH family RNA-binding protein Mmi1 recruits CPF to the noncanonical termination sites, promoting termination of these noncoding genes and facilitating heterochromatin assembly (Vo et al., 2019). Notably, interaction between CPF and Seb1 was observed (Lemay et al., 2016; Wittmann et al., 2017; Larochelle et al., 2018), providing additional evidence that termination-related factors play important roles in gene silencing.

In contrast to its homolog Nrd1 in budding yeast, S. pombe Seb1 is essential for transcriptional termination of not only noncoding genes but also coding genes (Wittmann et al., 2017). However, a follow-up study showed that binding of Seb1 to noncoding genes (such as dg and dh RNA) is substantially stronger than to coding genes as per portion of transcripts (Parsa et al., 2018). The authors proposed that, similar to Nrd1, Seb1 binds to these noncoding RNAs with some sequence specificity, although the consensus motif is very degenerate (Parsa et al., 2018). The exact mechanism of Seb1-promoted silencing is still largely unknown; however, as discussed below, current evidence suggests that Pol II pausing or the increased duration of transcript at the locus could be important for this silencing mechanism (Fig. 2A).

High-resolution mapping of Pol II position through Native Elongating Transcript sequencing data suggests that Seb1 also plays a general role in promoting Pol II pausing at the 5′ end of genes, including at centromeric repeats (Parsa et al., 2018). Loss of Seb1 leads to increased distribution of Pol II toward the gene body.

Figure 2. Parallel examples of cotranscriptional processing linked with chromatin gene repression. The graphs illustrate the roles of Seb1 and Paf1c in heterochromatin silencing in S. pombe. A, Seb1 is necessary for maintaining silencing in an RNAi-independent manner, involving its direct binding of RNA and Pol II pausing. B, Loss of Paf1c components leads to failure of RNA release from the locus and de novo establishment of a heterochromatic patch. WT, Wild type.
A link between Seb1-induced Pol II pausing and silencing was established by the study of the *tfs1* mutant. In this mutant, Pol II cannot resolve from a back-tracked state and therefore mimics the effect of increased Pol II pausing. Ectopic formation of H3K9me patches was observed in *tfs1* after loss of an antisilencing factor, Epe1, which promotes H3K9me turnover (Parsa et al., 2018). This experiment strongly supports cotranscriptional events such as Pol II pausing playing an important role in yeast RNAi-independent silencing (Fig. 2A).

In *S. pombe*, the Paf1c complex suppresses artificial small interfering RNA (siRNA)-induced ectopic gene silencing (Kowalik et al., 2015). A forward genetic screen was performed to identify mutations enhancing the function of siRNAs, which would otherwise be nonfunctional in the wild type (Kowalik et al., 2015). Mutants of all the Paf1c complex members were recovered in this screen. In the presence of exogenous siRNA, loss of Paf1c leads to de novo establishment of heterochromatin; such heterochromatic patches are self-sustainable through the RNAi-dependent silencing pathway and no longer require exogenous siRNA (Kowalik et al., 2015). Interestingly, loss of the termination factor Ctf1, but not the elongation factor Tfs1, has a similar effect to *paf1c* in facilitating silencing, suggesting that efficient termination prevents the siRNA-mediated formation of heterochromatin. Inefficient release of nascent transcripts from chromatin is important for the effect of Paf1c in promoting silencing (Kowalik et al., 2015). Therefore, the release of transcripts and/or efficient termination are important for preventing silencing (Fig. 2B).

The above studies highlight the role of transcript duration at chromatin in gene silencing, although the exact mechanism remains unclear. Given the known roles of FCA, FPA, and FY in transcription termination and the fact that *cdkc2* (an elongation factor) was recovered as a *sof* mutant, a related mechanism likely operates at the *FLC* locus. Indeed, FCA imposes both slow elongation rate and slow initiation rate at the *FLC* locus in its repressed state (Wu et al., 2016). The dura-
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Further strengthening this view (Bäurle and Dean, 2008), FLD contains an amine oxidase domain and is a homolog of the human H3K4me demethylase LSD1. FLD demethylates H3K4me2 (Liu et al., 2007; Wu et al., 2016) at the *FLC* gene body. In the repressed state, a low level of H3K4me2 at the *FLC* gene body is associated with a low level of H3K4me3 and H3Ac, especially at the first nucleosome of *FLC* (corresponding to *FLC* exon 1; Fig. 3A). The lowered H3K4me3 is likely due to absence of the COMPASS complex at *FLC* in its repressed state (Li et al., 2018). The lowered acetylation is linked with FLD (He et al., 2003), and FLD is associated with HDA6 deacetylase and FVE (Yu et al., 2016). Notably, knockout of HDA6 leads to a relatively weak delay of flowering (Yu et al., 2011). An mRNA sequencing analysis indicated that ~20% of misregulated genes in *fld* were also misregulated in *hda6* (Yu et al., 2016), suggesting some interplay between FLD and the deacetylation pathway. In addition to FLD, two other homologs of LSD1, LDL1 and LDL2, also target *FLC*, although they have relatively subtle effects on its expression (Jiang et al., 2007).

One histone mark enriched at *FLC* in its repressed state is H3K27me3, which covers the whole *FLC* locus from the transcription start site to the 3′ end (Fig. 3A). In autonomous pathway mutants, H3K27me3 at *FLC* is strongly reduced. As in *Drosophila melanogaster* and mammals, H3K27me3 in plants is catalyzed by the Polycomb Repressive Complex 2 (PRC2; Schubert et al., 2005; Margueron and Reinberg, 2011). The plant PRC2 can have different conformations, incorporating three closely related SET domain proteins all having H3K27me3 methyltransferase activity. Among these, CURLYLEAF (CLF) and SWINGER are mainly responsible for H3K27me3 during vegetative stages (Chanvivattana et al., 2004; Bouyer et al., 2011). Loss of CLF leads to reduction of H3K27me3 genome wide, accompanied by increased *FLC* expression (Lopez-Vernaza et al., 2012), although to a lesser extent than that observed in *fca* or *fpa* mutants (Lopez-Vernaza et al., 2012). Loss of FPA in *clf* significantly increases *FLC* expression level, so the relationship between PRC2 and FCA/FPA is still unclear (Lopez-Vernaza et al., 2012). A recent study showed that FCA has the potential to interact with CLF, suggesting a role of FCA in regulating H3K27me3 directly (Tian et al., 2019). However, such interaction was captured neither in FCA in vivo immunoprecipitation-mass spectrometry (Fang et al., 2019) nor in CLF in vivo immunoprecipitation-mass spectrometry (Liang et al., 2015). In addition, FCA functions genome wide in alternative polyadenylation and termination, where H3K27me3 is unlikely to be relevant (Sonmez et al., 2011). Thus, it is still unclear how autonomous pathway function establishes H3K27me3 at *FLC*. The cause and consequence of trans-factor functioning and chromatin modification are complex, as transcription is inhibited by H3K27me3, while recruitment of PRC2 and establishment of H3K27me3 can be a consequence of repressed transcription (Berry et al., 2017; Portoso et al., 2017; Laugesen et al., 2019).

**TUG OF WAR AT *FLC* CHROMATIN**

The fact that FLD is one of the strongest *sof* mutants suggests that FCA-mediated repression operates mainly at the chromatin level. *fld* also suppresses 35S:FPA,
The autonomous pathway can be seen as opposing the FLC activation up-regulation by FRI. Indeed, the relationship between FRI and the autonomous pathway is similar to a tug of war between active and repressed states (Fig. 3). In the presence of active FRI, FCA can win the game (and thus repress FLC) if it is overexpressed. In the 35S:FCA FRI background, FLC is efficiently repressed at the transcriptional level. In many winter annual accessions, however, FRI wins the game, with FLC being highly expressed and therefore conferring the requirement for vernalization (Clarke and Dean, 1994; Sända et al., 1997; Johanson et al., 2000). In this active state, FLC chromatin features high levels of H3K36me3, H3K4me3, and H3Ac, with low levels of H3K27me3 (Fig. 3B). The high expression level of FLC requires the H3K36me3 methyltransferase EFS (Soppe et al., 1999; Kim et al., 2005; Zhao et al., 2005; Xu et al., 2008; Hyun et al., 2017). The efs mutant is epistatic to fca, displaying early flowering with low FLC expression level (Soppe et al., 1999; Wu et al., 2016). High expression of FLC also at least partially requires Paflc complex components. Loss of function of Paflc components, such as VIP5, VIP6, and CDC73, leads to early flowering (Oh et al., 2004; Yu and Michaels, 2010), and the loss of CDC73 partially suppresses the high expression level of FLC in the fca and fy backgrounds (Yu and Michaels, 2010). The high expression of FLC in the presence of FRI requires the COMPASS-like complex, a conserved H3K4me3 methyltransferase complex (Jiang et al., 2009, 2011; Li et al., 2018). It is likely that mutants of COMPASS-like complex members suppress the fca phenotype, given the antagonism between FRI and the autonomous pathway.

The opposing chromatin states are the heart of the tug of war between FRI and autonomous pathway activity at FLC. These opposing chromatin states coordinately affect transcriptional initiation and elongation states, and these transcriptional outputs feed back to reinforce the opposing chromatin states (Fig. 3). In fca or fld mutants, transcriptional initiation and elongation are both up-regulated (25× and 10×) compared with the wild type, a process requiring EFS methyltransferase activity (Wu et al., 2016). This was demonstrated through mathematical modeling in combination with nascent RNA profiling along FLC intron 1. Given the rapid cotranscriptional splicing of FLC intron 1, altered elongation rate leads to unequal fold up-regulation of nascent RNA at the beginning and end of FLC intron 1 in fca (Wu et al., 2016). Using the same approach, FRI was shown to cause a similar coordination of transcriptional firing and elongation at FLC, associated with binding of histone acetyltransferases and the histone methyltransferase COMPASS-like (Li et al., 2018). However, the interconnectedness of cotranscriptional processes is very tight. RNA-binding proteins can play a direct role in transcription (Michelotti et al., 1996;
Kuninger et al., 2002; Ji et al., 2013; Xiao et al., 2019). For example, the mammalian splicing regulator SR protein SRSF2 regulates transcriptional elongation by controlling Pol II pause release at the 5’ end of a gene (Ji et al., 2013). RNA-binding proteins typically associate around transcription initiation sites (Xiao et al., 2019). In addition, chromatin association of transcription factors can depend on RNA-binding proteins (Xiao et al., 2019). An understanding of where different autonomous components associate with FLC will help establish how alternative processing of an antisense transcript delivers a chromatin environment that coordinately regulates transcriptional initiation and elongation.

**AUTONOMOUS PATHWAY COMPONENTS ASSEMBLE IN NUCLEAR BODIES THAT HAVE LIQUID-LIKE PROPERTIES**

The most recent sof mutant to be analyzed revealed a role for LLPS in the dynamic assembly of autonomous pathway components (Fang et al., 2019). Phase separation of proteins with similar biophysical properties generates biomolecular condensates, thus spatially compartmentalizing functions without the need for membrane boundaries (Banani et al., 2017; Shin and Brangwynne, 2017). Intracellular LLPS underlies the formation of dynamic membraneless organelles such as the nucleolus, Cajal bodies, and P-bodies. The driving forces of LLPS are multivalent intramolecular or intermolecular (protein-protein and protein-RNA) interactions (Li et al., 2012; Banani et al., 2017). Many of the proteins that can phase separate, particularly RNA-binding proteins, contain intrinsically disordered regions (IDRs; Molliex et al., 2015; Banani et al., 2017; Shin and Brangwynne, 2017). IDRs often have low sequence complexity and are characterized by polar residues that favor protein-protein interactions and/or isolated hydrophobic regions that drive aggregation (Banani et al., 2017; Shin and Brangwynne, 2017; Wang et al., 2018). A special class of IDRs are prion-like domains (PrLDs; Han et al., 2012; Wang et al., 2018). A pioneering bioinformatic analysis from Susan Lindquist’s group identified 474 Arabidopsis proteins with putative PrLDs (Chakrabortee et al., 2016), including the four autonomous pathway components LD, FCA, FPA, and FY. Further analyses showed that the PrLDs of LD, FPA, and FCA can form puncta in yeast cells (Chakrabortee et al., 2016). In addition, the PrLD of LD substituted for the PrLD of a known yeast prion, Sup35 (Chakrabortee et al., 2016).

FCA is predicted to contain two PrLDs at its C terminus and localize to multiple nuclear bodies. FCA nuclear bodies are extremely dynamic, with fast recovery times as measured by fluorescence recovery after photobleaching, and can fuse upon contacting each other, confirming that FCA undergoes LLPS in vivo (Fang et al., 2019). Interestingly, while the PrLDs of FCA readily undergo LLPS in vitro, full-length FCA alone is not sufficient, suggesting that there are additional regulators of FCA nuclear body formation in vivo (Fang et al., 2019).

The sof mutant, fl2, influences the formation of these FCA nuclear bodies (Fang et al., 2019). FLL2 is a coiled-coil protein that also contains PrLDs. Indeed, FLL2 forms nuclear bodies that overlap with those of FCA. More importantly, mutation of FLL2 reduces the size and number of FCA nuclear bodies, revealing a role of coiled-coil domains in facilitating LLPS of FCA (Fang et al., 2019). Given the property of coiled-coil domains in mediating protein-protein interactions and oligomerization, it is tempting to hypothesize that through its self-oligomerization and interaction with FCA, FLL2 and its paralog(s) increase the local concentration of FCA, which then leads to LLPS and nuclear body formation (Fig. 4).

Similar to other sof mutants, fl2 also reduces the proximal-to-distal polyadenylation ratio of COOLAIR, implying that FCA nuclear bodies promote proximal COOLAIR 3′-end processing (Fang et al., 2019). To further understand the functionality of FCA nuclear bodies,
the authors adopted a technique called cross-linked nuclear immunoprecipitation and mass spectrometry, in which they cross-linked plants with formaldehyde and immunopurified FCA for mass spectrometry analysis. Components of the 3′-end processing machinery copurified with FCA, including FPA, FY, and other proteins from the polymerase and nuclease modules of the canonical 3′ RNA processing complex (Fig. 4). Most of the copurified 3′ processing factors colocalized with FCA in nuclear bodies. These data unambiguously support the idea that nuclear bodies formed by LLPS of FCA are key to 3′-end processing of certain transcripts, including COOLAIR. It remains to be determined whether nuclear bodies are the sites of COOLAIR processing and polyadenylation, or in other words, whether the nuclear bodies colocalize with the FLC locus or COOLAIR nascent transcripts. Furthermore, given that FCA and FPA are RNA-binding proteins, the role of RNA in nuclear body formation will be interesting to explore in the future.

CONCLUSIONS AND FUTURE PROSPECTS

Investigation of the autonomous pathway, apparently specific to flowering time regulation, has uncovered a cotranscriptional silencing mechanism with functions throughout the Arabidopsis genome and parallels in many organisms. An interesting, and yet unresolved, question is why mutations in this genome repression pathway have phenotypes specific to flowering time. We favor loss of redundant cotranscriptional regulators specifically at FLC to explain this paradox. An important unsolved question (see Outstanding Questions) is how cotranscriptional RNA-processing events are linked to the downstream chromatin mechanisms. At FLC, this translates into how cotranscriptional events mediated by FCA, PRP8, and CDKC2 are linked to FLD-mediated chromatin repression. Additional sof mutant screening would likely provide answers in an unbiased way. Detailed functional analysis of LD and FLK will also be valuable, given their importance as members of the autonomous pathway. In addition, further understanding of autonomous pathway components at the whole-genome level will be informative. The recruitment mechanism of these proteins remains unknown. It is also unclear if they have a general role in transcription, as is the case of Srb7 in fission yeast. The application of Native elongating transcript sequencing (Nojima et al., 2015; Zhu et al., 2018) and UV cross-linking and immunoprecipitation sequencing (König et al., 2010; Zhang et al., 2015; Meyer et al., 2017) in autonomous mutants would likely help elucidate the answers to these important questions. Furthermore, the use of single-cell technologies and chromatin interaction analyses, such as has been undertaken in the 4D Nucleome Project (Dekker et al., 2017), would provide information on how these factors coordinate and cooperate with each other with high temporal and spatial resolution. The progress made from these future attempts will aid our understanding of cotranscriptional silencing mechanisms.

ACKNOWLEDGMENTS

We thank Congyao Xu, Mathias Nielsen, and Deyue Yang for helpful comments on the article.

Received August 16, 2019; accepted October 28, 2019; published November 18, 2019.

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