OUTLOOK

Winter fields antisense RNAs to kick off flowering

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FLOWERING LOCUS C (FLC), a MADS-box transcription factor, plays a major role in determining flowering time in *Arabidopsis*. In this issue of *Genes & Development*, Zhao and colleagues (pp. 888–898) elucidate the role of *COOLAIR* antisense noncoding RNAs in *FLC* regulation through field trials and laboratory experiments. *COOLAIR*-mediated *FLC* silencing is induced by the first seasonal frost in the field and thus acts as a key molecular indicator during autumn for winter arrival.

Temperature is a major seasonal cue for plants to align flowering time. High activity of the central floral repressor FLC inhibits untimely flowering in the model plant *Arabidopsis* and also crop species (Michaels and Amasino 1999; Takada et al. 2019). A thorough dissection of *FLC* regulation in *Arabidopsis* identified an impressive collection of contributing mechanisms (Wu et al. 2020). A tantalizing observation is that defects in conserved factors regulating RNA polymerase II (RNAPII) transcription often result in seemingly specific flowering time phenotypes through altered *FLC* expression. Allelic variation at *FLC* in *Arabidopsis* accessions collected around the world highlighted DNA sequence polymorphisms at this locus to the local adaptation of flowering (Hepworth et al. 2020). A fascinating aspect of *FLC* expression regulation is the responsiveness to long cold periods (winter) that trigger PRC2-based chromatin silencing to ensure low *FLC* expression when conditions are favorable in spring.

Like many other genes in *Arabidopsis*, several transcript isoforms can be generated from the *FLC* locus (Thomas et al. 2020). Perhaps surprisingly, *COOLAIR*, a group of long noncoding antisense RNAs expressed from the *FLC* locus, emerged as central coordinator of *FLC* expression. The discovery of *COOLAIR* provided a previously missing target for pathways mediating alternative polyadenylation (Liu et al. 2010). Moreover, cold results in *COOLAIR* induction, offering a molecular rationale for how high *FLC* expression can be reversed by cold to allow flowering. Mutations in 3′ end formation complexes flower extremely late due to high FLC activity, but cold treatment and resulting *COOLAIR* induction can substitute for 3′ end formation defects and accelerate flowering (Koornneef et al. 1991). *COOLAIR* thus provides the *FLC* locus with a neat cold sensor to initiate repression in preparation for spring. *COOLAIR* induction in laboratory conditions peaks after 2–3 wk of cold exposure, but induction can be detected within hours after cold treatment by methods detecting nascent RNAPII transcription (Kindgren et al. 2020). These findings raised the question of how *COOLAIR* activity is triggered by natural environments.

Zhao et al. (2021) clarify *FLC* regulation in natural temperature regimes. The investigators assayed the expression of *FLC* and *COOLAIR* isoforms in samples from key selected accessions grown in three field sites across Europe. Field trial data revealed that *COOLAIR* is highly induced when plants experience a dip below freezing temperature, likely analogous to the first frost in autumn. The freezing-induced *COOLAIR* spike coincides with the reduction of *FLC* transcripts. Importantly, controlled environment chambers that recapitulate natural temperature regimes could validate these findings, and show that a freezing-induced *COOLAIR* spike represents a key trigger for *FLC* repression. These findings support the view that the *COOLAIR-FLC* circuitry of repression is triggered by an exposure to first frost in natural temperature regimes, and that this property likely underpins environmental adaptation.

This study presents an authoritative clarification of the functional role of *COOLAIR* in *FLC* repression. Testing the function of lncRNA represents a formidable challenge, since it is often unclear what aspect of the lncRNA needs to be disrupted to reveal the key activity (Bassett et al. 2014). Among lncRNAs, the functional characterization of antisense lncRNA is a particularly tough nut to crack since mRNA and antisense lncRNA are generated

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from the same DNA template. The opportunities for mutational approaches targeting transcription of the antisense DNA strand specifically are thus severely limited, in particular in experimental systems such as *Arabidopsis*, where knock-in mutational approaches are not commonly available.

Zhao et al. (2021) offer an impressive collection of molecular approaches to alter *COOLAIR* expression. These include two strategies to achieve *COOLAIR* overexpression in *cis*. They first isolated T-DNA insertion mutants that result in *COOLAIR* overexpression, and second, dominant mutations (*ntl8-D3*) of the transcriptional regulator of *COOLAIR*, NTL8 (Zhao et al. 2020). Quantitative single-cell imaging methods establish that *COOLAIR* induction anticorrelates with unspliced *FLC* transcript levels (used as a proxy for transcription), strongly supporting a role in repression. The inverstigators describe five experimental strategies to engineer specific *COOLAIR* loss-of-function mutants. Four of these rely on a two-step approach based on the *flc-2* fast neutron mutation where most *FLC* sequences have been deleted. The *flc-2* mutant is then complemented with genomic clones of *FLC* where sequences in the 3′ end linked to transcriptional termination of *FLC* mRNA and the induction of *COOLAIR* are replaced. The fifth strategy relies on CRISPR/Cas9 deletion of sequences in the *FLC* terminator region that include the transcription start sites (TSSs) of some *COOLAIR* isoforms. A comprehensive analysis of *FLC* and *COOLAIR* transcription in this collection of mutants aiming to eliminate *COOLAIR* expression showed that these strategies were most effective for isoforms initiating close to the mutated sequences. However, none of the strategies fully eliminated distal *COOLAIR* transcription overlapping the TSSs of *FLC* mRNA. Targeted analysis of TSSs in *COOLAIR* mutants showed that novel antisense transcript isoforms are generated when initiation of *COOLAIR* is repressed. A hotspot of TSSs for the novel antisense transcript isoforms is the first intron of *FLC*, reminiscent of *convergent antisense species* (*CAS*), previously identified by genome-wide analyses in *Arabidopsis* (Kindgren et al. 2020). These analyses provide detailed insight into the fluidity of antisense transcript isoforms that may have contributed to prior confusion about the functional role of *COOLAIR* in *FLC* repression when only a selection of mutants was analyzed. Double mutants between *ntl8-D3* and *COOLAIR* mutants showed that *FLC* reduction by *ntl8-D3* requires the generation of *COOLAIR*, arguing for some functional specification of *COOLAIR*.

In conclusion, careful and holistic analyses of temperature-sensitive *FLC* expression from the field to the laboratory illustrates how the interplay between mRNA and lncRNA isoforms results in chromatin-based regulation of plant gene expression. This study offers insight into how temperature-responsive gene expression is achieved by plants, which should offer inspiration for strategies aiming to promote food security in a changing climate.

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