The intersection of DNA replication with antisense 3′ RNA processing in *Arabidopsis* FLC chromatin silencing

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How noncoding transcription influences chromatin states is still unclear. The *Arabidopsis* floral repressor gene FLC is quantitatively regulated through an antisense-mediated chromatin silencing mechanism. The FLC antisense transcripts form cotranscriptional R-loop that is dynamically resolved by RNA 3′ processing factors (FCA and FY), and this is linked to chromatin silencing. Here, we investigate this silencing mechanism and show, using single-molecule DNA fiber analysis, that FCA and FY are required for unimpeded replication fork progression across the *Arabidopsis* genome. We then employ the chicken DT40 cell line system, developed to investigate sequence-dependent replication and chromatin inheritance, and find that FLC R-loop sequences have an orientation-dependent ability to stall replication forks. These data suggest a coordination between RNA 3′ processing of antisense RNA and replication fork progression in the inheritance of chromatin silencing at FLC.

DNA replication | transcription | chromatin silencing | *Arabidopsis* | chicken DT40

**genetic analysis** identified FCA (an RNA binding protein) and FY (a component of the cleavage polyadenylation specificity factor complex) as components of an antisense-mediated chromatin silencing mechanism at *Arabidopsis* FLC (1). Their 3′ processing activities have widespread roles in transcriptional termination in the *Arabidopsis* genome (2). At FLC, they resolve an NDX-stabilized R-loop generating a proximally polyadenylated COOLAIR transcript (3–6). Resolution of the R-loop recruits chromatin modifiers, including the H3K4me1 demethylase FLD, required for low FLC expression (7). FLD interacts with the SET domain protein SDG26, which, in turn, transiently interacts with FY, providing a physical link between R-loop resolution and chromatin silencing at FLC (4). These activities are part of the autonomous floral pathway, which promotes rapid flowering in accessions such as *Columbia*, and are completely independent of the cold-induced Polycomb silencing mechanism in the vernalization process. The connections between R-loop resolution and chromatin inheritance at FLC remain poorly understood.

Recent work in mammalian cells has shown that RNA 3′ processing is linked to DNA replication (8). Cotranscriptional R-loops have also been implicated in impeding replication fork progression to impact epigenetic inheritance (9). Replication/transcription collisions have been proposed to control chromatin states (10). We, therefore, set out to test whether FY- and FCA-mediated RNA 3′ processing interferes with ongoing *Arabidopsis* replication forks. We developed a single-molecule DNA combing assay for *Arabidopsis*, based on the in vivo incorporation in seedlings of the thymidine analog CldU and the stretching of individual DNA fibers onto glass slides (*SI Appendix*). Progression of replication forks was tested at the genomic level in wild-type (WT) Col-0, fy-2, and fca-9 *Arabidopsis* mutant seedlings. A statistically significant reduction in the overall length of pairs of replication forks was observed in fy-2 compared to Col-0 and fca-9 (Fig. 1A). Closer examination of individual fork pairs revealed that, in Col-0, most replication forks were symmetric, with the left and right forks departing from the same origin having the same length of replicated DNA, indicating they both progress unimpeded. In contrast, in fy-2 and, to a lesser extent, in fca-9, replication forks were found to be asymmetric, suggesting that one of the diverging forks is being stalled (Fig. 1B and C). The lower frequency of asymmetric forks in fca-9 compared to that in fy-2 is likely to be explained by the fact that FCA appears to function at only a subset of FY targets (2). These results indicate that defects in *Arabidopsis* 3′ RNA processing impact replication fork progression.

We then wanted to understand whether FCA and FY processing of the FLC R-loop influences replication fork progression. This is technically not feasible in planta, so we exploited the chicken DT40 cell line, which provides a well-characterized system to assay the effect of heterologous sequences on progression of the replisome (9, 11). Sequences are cloned downstream of the chicken BU-1 transcription start site, and their ability to confer expression instability to BU-1 expression is monitored by fluctuation analysis. Such instability manifests when sequences cloned onto the leading strand form secondary structures that significantly stall the replisome, causing defective histone redeposition and the consequent loss of epigenetic information. Sequences cloned onto the lagging strand, instead, have been shown not to cause instability (9, 11, 12). To test whether the FLC 3′ sequences influence replisome progression, we cloned an 815-bp FLC fragment, which corresponds to the R-loop–forming sequences, into the BU-1 locus, in both orientations (Fig. 2A). As experimental controls, we used cells containing a previously characterized poly-purine (GAA)10 repeat at the same locus, which forms a triplex DNA structure and an R-loop (9), or cells without any predicted structure at the BU-1 locus (WT ΔG4). Similar to the (GAA)10 repeat construct, the FLC sequences caused no significant changes in BU-1 expression stability in WT cells (Fig. 2B). However, replisome stalling may be masked by mechanisms that restart DNA replication, such as the primase-polymerase PrimPol, which reprimps replication downstream of fork stalling structures; PrimPol loss increases the probability of a leading strand stall leading to disruption of the BU-1 epigenetic state (9, 11). Therefore, to sensitize the system, we tested a primpol mutant line and found a modest, but not statistically significant, increase in BU-1 instability when the FLC coding strand undergoes leading strand replication. However, in the orientation where the FLC template strand undergoes leading strand replication, BU-1 instability was pronounced (Fig. 2B). Structure predictions of the FLC 3′ sequences suggest
possible triplex-forming and G-quadruplex-forming regions (see SI Appendix). Furthermore, the FLC sequences remain capable of forming R-loops, as shown by RNase H-sensitive S9.6 antibody immunoprecipitation (Fig. 2C), and R-loop formation contributes to BU-1 instability in primpol cells overexpressing RNase H1 (Fig. 2D). The use of the DT40 system thus identified sequences on the template strand of FLC that effectively stall the replisome in an R-loop–dependent manner.

Together, our data allow us to propose a model whereby, in planta, FCA and FY recognize an NDX-stabilized R-loop and efficiently terminate the COOLAIR transcript to resolve the R-loop. These R-loop dynamics would influence formation of a replisome-stalling structure on the single-stranded DNA strand opposite the R-loop. In fca or fy mutants, where the R-loop is not resolved, the structure would form and stall the replication fork, and SDG26, FLD-mediated chromatin silencing would fail to be propagated (Fig. 2E). In WT cells, we envisage that the R-loop dynamics cause replisome slowing, but not stalling, creating a temporal window that facilitates recruitment and activity of FLD (Fig. 2E). Epigenetic silencing mediated by LSD1, the homolog of FLD, has been linked to R-loop homeostasis in mammalian cells (13). LSD1 is also involved in replication fork pausing at the mating-type locus in fission yeast (14), and the Rik1 complex, which associates with the replisome, silences heterochromatin via the H3K4me3 demethylase Lid2 (10). These and our findings suggest the importance of the interplay between RNA 3’ processing, R-loop stabilization, and replication fork progression in chromatin inheritance.

Materials and Methods

Detailed descriptions are provided as SI Appendix.

For the Arabidopsis DNA replication/combing assay, seedlings were in vivo labeled with CldU, nuclei were extracted and embedded in agarose blocks, DNA was deproteinized, and the DNA fibers were stretched onto glass coverslips. Incorporated CldU and DNA fibers were immunodetected, and images were analyzed using Fiji software.

For the DT40 cell culture replication assay, the 3’ FLC region was inserted into the BU-1 locus by cloning the region into the BU-1 targeting vector, and transfecting into DT40 cells; successful insertion was determined by puromycin selection and flow cytometry. Overexpression of RnaseH1 was achieved by viral transduction of chicken RNaseH1 and RnaseH1 expression established by flow cytometry. For BU-1 fluctuation analyses, cells were grown for 20 generations.
and stained with anti-BU-1a antibodies, and clones were analyzed by flow cytometry for the percentage of cells that had lost expression.

**Data Availability.** All study data are included in the article and SI Appendix.

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