Supporting Information

SI Material and Methods

RNA extraction and qRT-PCR

RNA extractions were done using the classical phenol-chloroform method. Total RNA samples were treated with Turbo DNA-free (Ambion) to remove any contaminating genomic DNA. The first strand of cDNA was prepared by Superscript III first strand synthesis system (Invitrogen) using gene specific primers. qPCR was performed on a Lightcycler 480 (Roche) with SYBR green Jumpstart reaction regent (Sigma) and analyzed for absolute quantification against a standard curve for each primer pair. The templates used to generate the standard curve were serial dilutions of cDNA. Every PCR was repeated at least twice while PCR for standard curve was repeated three times.

Cordycepin treatment

14 days-old non-vernalized samples and 12 days vernalized samples (10 days in NV condition plus 2 weeks in cold) were prepared. 12 seedlings of similar size/ physiological state were used for each time-point. Seedlings were transferred from GM plates into sterile incubation buffer (1 mM PIPES pH 6.25/ 1 mM sodium citrate/ 1 mM potasium chloride/ 15 mM sucrose) (35) and preincubated for 30 minutes with 75 rpm swirling. After preincubation cordycepin was added to the buffer for 100 μg/ml final concentration. Samples at given time-points were taken, blotted dry, frozen in nitrogen and kept at -80°C. Each treatment was done in three replicates. RNA was purified by phenol extraction method. DNase treated (Turbo DNase, Ambion) RNAs were used to do specific-priming RT reactions for sense and antisense transcripts (primers sequences are listed in Table S2). qRT-PCR reactions were done using Sybrgreen (Life Technologies). Relative amount of transcripts were calculated based on the Ct values (for *FLC* unspliced and mRNA, *COOLAIR* total RNA and eiF4A as an internal control). Relative amount of *FLC* and *COOLAIR* transcripts was normalized to eiF4A control for each corresponding time-point first then normalized to the 0 time point (no cordycepin value). Values are means +/- S.E.M of three biological replicates.

Nuclear and Cytoplasmic RNA extraction

Plant material was ground in liquid nitrogen, the powder resuspended in ice-cold wash buffer (20 mM Tris-HCl pH7.5, 2.5 mM magnesium chloride, 25% glycerol). The material was clarified with filtration twice through Miracloth, then pelleted at 1,000 rpm, 4°C for 5 minutes. The resuspension was repeated twice in fractionation buffer supplemented with 0.3% triton X100 (20 mM Tris-HCl pH7.5, 2.5 mM magnesium chloride, 25% glycerol, 0.3% triton X100) to lyse cells and organelles but keep nuclei intact. The material was centrifuged at 4°C 1,000 rpm, for 10 minutes. Supernatants were retained as cytoplasmic fraction. The nuclear pellet was resuspended in Lysis Buffer (20 mM Tris-HCl pH 7.5, 200 mM potasium chloride, 2 mM DTT, 1% triton X-100). RNA was extracted from the cytoplasmic and nuclear fractions with phenol/chloroform, precipitated in ethanol. RNA elutions were used in qRT-PCR to measure the content of different RNA species. U6 and tRNA were used as internal controls for nuclear and cytoplasmic extracts respectively. U6 nuclear marker shows that the cytoplasmic fraction is contaminated with nuclear content. tRNA cytoplasmic control indicates that the nuclear fractions are highly pure and free of cytoplasmic contaminants.

ChIRP protocol

Antisense DNA probes were designed against *COOLAIR* spliced forms using an online designer at http://www.singlemoleculefish.com (see primer locations on Fig. 2D and primer sequences in Table S1). All probes were biotinylated at the 3' end with an 18-carbon spacer arm (Protein and Nucleic Acid Facility, Stanford University). Plant material was collected at 2 weeks non-vernalized (NV) or 2 weeks vernalized (2w) time-points. Crosslinking was performed in the presence of 1% glutaraldehyde in PBS for 10 minutes at room temperature and then quenched with 0.125 M glycine for 5 minutes. Crosslinked plant material was washed, blotted dry and frozen in liquid nitrogen. Chromatin extraction was done as for ChIP experiments. Chromatin was sonicated until DNA was in the size range of 100–500 bp. Probe hybridization to sheared chromatin was done in the hybridization buffer (750 mM sodium chloride, 1% SDS, 50 mM Tris-HCl 7.0, 1 mM EDTA, 15% formamide. DTT, PMSF, Protease Inhibitor Complete tablets (Roche), and Superase-in were added to the buffer prior to use). Probes of 100 pmol were added to 1.5 ml of diluted chromatin, mixed by end-to-

end rotation at 37°C for 4 hr. 100µl of washed C1 beads (Dynabeads) were added per 100 pmol of probes, and the whole reaction was mixed for another 30 min at 37°C. Beads:biotin-probes:RNA:chromatin adducts were collected on magnets, washed 5 times with 50x bead volumes of wash buffer (2XSSC, 0.5% SDS, DTT and PMSF added freshly). RNA was extracted after proteinase K treatment with phenol:chloroform extraction and precipitated in ethanol. Eluted RNA was subject to quantitative reverse-transcription PCR (qRT-PCR). Bound DNA was eluted from beads using RNaseA/H mixture (Sigma-Aldrich) in DNA elution buffer (50 mM $NaHCO_3$, 1% SDS, 200 mM sodium chloride). RNase elution was carried out twice at 37°C with end-to-end rotation and eluent from both steps was combined. The eluted DNA was proteinase K treated, extracted with phenol:chloroform and precipitated in ethanol. Eluted DNA was subject to qPCR or high throughput sequencing.

RNA Immunoprecipitation

Without cross-linking, plants were ground and nuclei were enriched as for ChIP. Pellet was resuspended in Lysis Buffer (50 mM Tris pH 7.5, 1 mM EDTA pH 8.0, 0.5% NP-40, 150 mM sodium chloride and 1 mM PMSF/Protease inhibitor Complete tablets (Roche). Lysates were sonicated on ice for 10 seconds (3 times), and centrifuged at top speed twice (14,000 rpm) for 5 minutes at 4°C. 10% aliquot was preserved as an input sample and frozen at -80°C. GFP-trap beads (20µl, Chromotek) were added to each tube containing 1 ml cleared lysate and each sample was incubated on a rotating mixer wheel for 2h at 4°C to collect immune complexes. Each immune complex was washed 5 times using low salt (dilution buffer), high salt (dilution buffer supplemented with sodium chloride to a final concentration of 500 mM). After washing, immune complexes were eluted by addition of 2XPK buffer (100 mM Tris-HCl pH 7.5, 200 mM sodium chloride, 2 mM EDTA, 1% SDS). RNA was purified using phenol-chloroform method. DNA from the samples was removed by using Turbo-DNase (Ambion). First strand cDNA was synthesized using strand specific primer mix (primers are listed in Table S2) and Superscript III (based on manufacturer's instructions, Invitrogen). RT products were amplified using the SybrGreen qPCR Master kit (Lifesciences). The relative fold changes of retrieved RNAs were calculated as followings: First, relative enrichments of sense or antisense RNA were calculated by comparison to the INPUT on each time-point. Second, relative levels of retrieved RNA for sense or antisense were calculated by comparison

to the relative enrichments of upstream region (-158F/-56R relative to ATG of *FLC* sense transcript) as a background level. As a RT control, reverse transcription was performed without Superscript III enzyme.

Supplementary material for figures

Fig. S1. RNA stability assays of *FLC* unspliced RNA, mRNA and *COOLAIR*. (A) Cold effect on the stability of *COOLAIR* ($T^{1/2}_{NV}$=100min, $T^{1/2}_{2w}$=240min). (B) Unspliced *FLC* RNA is slightly stabilized in cold ($T^{1/2}_{NV\ uFLC}$=80min, $T^{1/2}_{2w0\ uFLC}$=178min. (C) *FLC* mRNA has a half-life in the range of days both at NV and 2w. (D) Unspliced *FLC* transcript stability is not affected in *TEX* construct ($T^{1/2}_{CTL}$=72min, $T^{1/2}_{TEX1}$=88min). (E) *FLC* mRNA half-life is only slightly affected by the absence of *COOLAIR*. Values are means +/- S.E.M of three biological replicates. (F) Hexamer elements that may contribute to *FLC* mRNA stability present in the 3' UTR of the RNA (based on (17)).

Fig. S2. *COOLAIR* accumulation is impaired in *TEX* transgenic lines**.** Relative induction of *COOLAIR* Class I (A), Class II (B) and unspliced forms (C) in representative single *CTL* and *TEX* (*TEX1*) lines. *COOLAIR* levels are the same as the *flc-2* background in *TEX1* and have lost all cold induction (A-C). Values are relative to NV levels in *CTL* lines (NV was set as 1). (D) *FLC* mRNA down-regulation in non-vernalized material (NV), after 1 week cold exposure (1w), 2 weeks cold (2w), 3 weeks cold (3w), 4 weeks cold (4w). Values are means +/- S.E.M of three biological replicates.

Fig. S3. Comparison of *COOLAIR* and *FLC-RBCS* antisense transcripts in *TEX* lines. Expression of Class I *COOLAIR* (A), unspliced *FLC* transcript (B), antisense transcripts from *FLC-RBCS* (C) in *CTL*, *TEX1* and *flc-2*. The position of the primers are depicted in Figure 1*B,* sequences are listed in the Table S2. For antisense RNA transcripts a combination of FLC and RBCS primers was used (see Table S2). Non-vernalized (NV), 1 week cold (1w), 2 weeks cold (2w), 3 weeks cold (3w), 4 weeks cold (4w). Values are means +/- S.E.M of three biological replicates.

Fig. S4. *COOLAIR* does not cause *FLC* repression through a transcriptional interference mechanism between sense and antisense transcription. Difference plot of

hybridizations of RNA from 2 weeks cold-treated and non-vernalized seedlings, hybridized to custom tiling array of both strands of the *FLC* locus arrayed at single nucleotide resolution (described in ref. 11). The array data is an average of three biological replicates.

Fig S5. Relative subcellular (nuclear or cytoplasmic) distributions of *FLC* transcripts. (A) U6 RNA as nuclear RNA control (relative value set to 1 in nuclear fraction), (B) Glycine tRNA as the cytoplasmic RNA control (relative value set to 1 in cytoplasmic fraction), (C) total *COOLAIR,* (D) *COOLAIR* Class I*,* (E) unspliced *FLC* and (F) *FLC* mRNA.

Fig. S6. Landscape view of ChIRP-seq data in the 50kb region around the *FLC* locus. *FLC* gene (At5g10140) is highlighted in yellow. Transcript organization and protein gene models are depicted above.

Fig. S7. qRT-PCR and qPCR validation of ChIRP data (Fig. 2 and S6) on non-vernalized (NV) and cold-treated (2w) plants. Class I (A) and class II *COOLAIR* (B) but not *UBC* mRNA (C) is efficiently enriched by COOLAIR-ChIRP. "i" stands for "input samples", nv (non-vernalized), 2w (2weeks cold treatment), rnase (RNase A/H mix treatment), PrK (Proteinase K treatment) (see also Materials and Methods). *COOLAIR* binds to *FLC* genomic DNA in two distinct regions (D). *COOLAIR* binding to *FLC* chromatin is affected after RNaseA/H or Proteinase K treatment. Numbers show the location of primers used in the qPCR relative to *FLC* translational start (ATG) site.

Fig. S8. Immunoprecipitation of components of the PHD-PRC2 complex does not enrich for *FLC* or *COOLAIR* RNA. (A) Immunoprecipitation of VRN5-eYFP and SWN-eGFP protein from NV and 2 weeks cold treated samples. (B) Schematic representation of primers location relative to *FLC* locus. Unspliced sense *FLC* (C and E) or antisense *COOLAIR* (D and F) enrichment in the VRN5-eYFP or SWN-eGFP pull-down samples respectively is at background levels both NV and at 2 weeks cold (2w). Relative enrichments were calculated based on control NV values. Error bars are calculated from two biological replicates except for (E) where a representative experiment is shown.

Fig. S9. *COOLAIR* presence is important for H3K4me3 down-regulation at *FLC*. ChIP analysis of H3K4me3 in wild-type Ler (A), *vrn5-5* (B), *TEXp* lines (C) and *vrn5-5*/*TEX1* (D).

Fig. S10. *COOLAIR* transcripts are up regulated in *vrn5-5* mutants compared to wild type (*Ler* FRI). Dynamics of cold-induction is not affected in *vrn5-5*. In *Ler* FRI ecotype Class II *COOLAIR* is not induced by cold treatment (opposite to *Col* FRI ecotype). Class I (A), Class II (B) and total *COOLAIR* (C) are at very low levels in TEX or vrn5/TEX. The position of the primers used are depicted in Fig 1*B*. For antisense RNA transcripts in TEX an FLC and RBCS primer combination was used. Primer sequences are listed in the Table S2. Values are means +/- S.E.M of three biological replicates.

Fig. S11. Flowering time analysis in control (*CTL*) and *TEX* pooled samples. Days to flower recorded without vernalization (NV) and after 1, 2, 4, 6 and 8 weeks of cold treatment. Values are means +/- S.D.

Supplementary tables

Table S1. Biotinylated DNA probes used for *COOLAIR*-ChIRP

tcgtgtgagaattgcatcga
ccaagagactttgtgtgtga
ggcgagcgtttgtatatctt
ggcggttgaaatcaaaatcc
ccactacttaattagccacc
aaatctccgacaatcttccg
aagctgagatggagatgtca
ggttgttatttggtggtgtg
agatggagatgtcacctgct
atcatcatgtgggagcagaa
gattatcgtacagatggaga
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tgtgctcttttacttttctt
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ttccttacctgggttttcat
tctcctccggcgataagtac
ttgtgctcttttacttttct
ctcttttacttttctgctgg

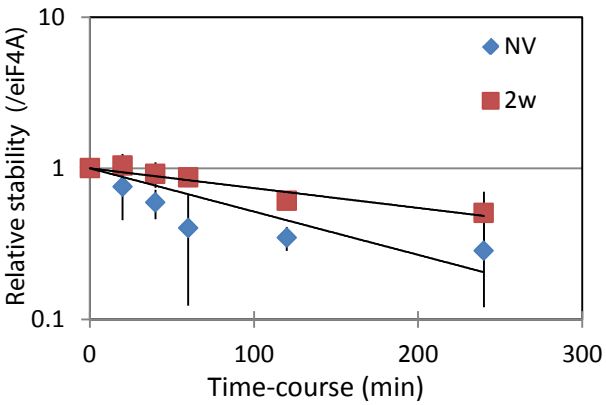
ctttccttacctgggttttc

Table S2. List of primers.

Experiment	Name	Sequence
ChIP	-2429_F	ATCCAGAAAAGGGCAAGGAG
	-2376_R	CGAATCGATTGGGTGAATG
	-1708_F	TGGAGGGAACAACCTAATGC
	-1639_R	TCATTGGACCAAACCAAACC
	-501_F	ACTATGTAGGCACGACTTTGGTAAC
	-381_R	TGCAGAAAGAACCTCCACTCTAC
	-158_F	GCCCGACGAAGAAAAAGTAG
	-56_R	TCCTCAGGTTTGGGTTCAAG
	48_F	CGACAAGTCACCTTCTCCAAA
	205_R	AGGGGGAACAAATGAAAACC
	307_F	GGCGGATCTCTTGTTGTTTC
	393_R	CTTCTTCACGACATTGTTCTTCC
	393_F	GGAAGAACAATGTCGTGAAGAA
	547_ R	GCACGCATCAGATCGTATCA
	679_F	TCATTGGATCTCTCGGATTTG
	817_R	AGGTCCACAGCAAAGATAGGAA
	543_F	CGTGCTCGATGTTGTTGAGT
	700_R	TCCCGTAAGTGCATTGCATA
	817_F	TTCCTATCTTTGCTGTGGACCT
	997_R	GAATCGCAATCGATAACCAGA
	1035_F	CCTTTTGCTGTACATAAACTGGTC
	1148_R	CCAAACTTCTTGATCCTTTTTACC
	1424_F	TTGACAATCCACAACCTCAATC
	1561_R	TCAATTTCCTAGAGGCACCAA
	1884_F	AGCCTTTTAGAACGTGGAACC
	2062_R	TCTTCCATAGAAGGAAGCGACT
	2356_F	AGTTTGGCTTCCTCATACTTATGG
	2451_R	CAATGAACCTTGAGGACAAGG
	3088_F	GGGGCTGCGTTTACATTTTA
	3224_R	GTGATAGCGCTGGCTTTGAT
	3899_ F	CTTTTTCATGGGCAGGATCA
	4088_ R	TGACATTTGATCCCACAAGC
	4213_F	AGAACAACCGTGCTGCTTTT
	4360_R	TGTGTGCAAGCTCGTTAAGC
	5030_F	CCGGTTGTTGGACATAACTAGG
	5135_R	CCAAACCCAGACTTAACCAGAC
	5534_F	TGGTTGTTATTTGGTGGTGTG
	5649_R	ATCTCCATCTCAGCTTCTGCTC
	5730_F	CACCTTAAATCGGCGGTTG
	5814_R	TACAAACGCTCGCCCTTATC

	5948_F	CGTGTGAGAATTGCATCGAG
	6066_R	AAAAACGCGCAGAGAGAGAG
	6416_F	AACAGTTCCAGACGCCATTG
	6488_R	TCATCTTGTAGGCTGAATCAGAG
	STM exon F	GCCCATCATGACATCACATC
	STM exon R	GGGAACTACTTTGTTGGTGGTG
	ACTIN 16_F	GATATTCAGCCACTTGTCTGTG
	ACTIN 100_R	CTTACACATGTACAACAAAGAAGG
qPCR	UBC qPCR F	CTGCGACTCAGGGAATCTTCTAA
	UBC qPCR R	TTGTGCCATTGAATTGAACCC
	FLC_qPCR_F	AGCCAAGAAGACCGAACTCA
	FLC_qPCR_R	TTTGTCCAGCAGGTGACATC
	FLCunspliced_F	CGCAATTTTCATAGCCCTTG
	FLCunspliced_R	CTTTGTAATCAAAGGTGGAGAGC
for Class I *COOLAIR*	Set2 new LP	TCATCATGTGGGAGCAGAAG
for Class I *COOLAIR*	Set2 new RP	TCTCACACGAATAAGGTGGCTA
for antisense transcript in *TEX*	5645F	GTGGGAGCAGAAGCTGAGAT
for antisense transcript in *TEX*	RBCS3-R	ACTTAATTGGCATTGAACCGGTAAC
for Total *COOLAIR*	Set6_new LP	TGTATGTGTTCTTCACTTCTGTCAA
for Total *COOLAIR*	Set6_new RP	GCCGTAGGCTTCTTCACTGT
for Class II *COOLAIR*	Set4_new F	GTATCTCCGGCGACTTGAAC
for Class II *COOLAIR*	Set4_new R	GGATGCGTCACAGAGAACAG

a



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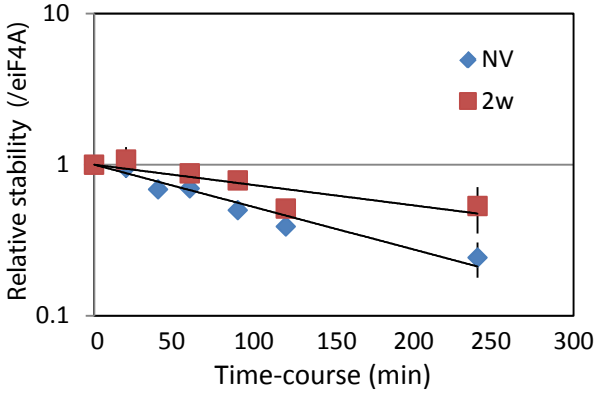

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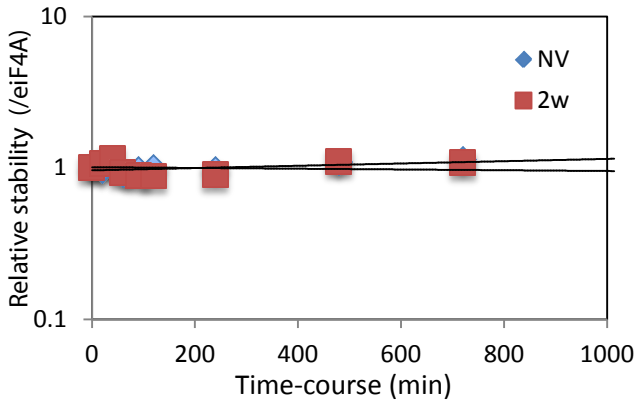

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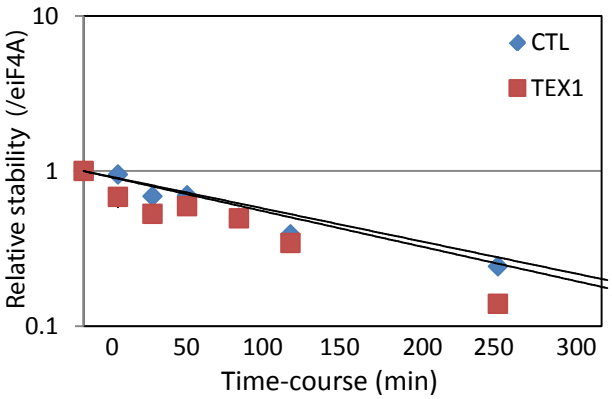

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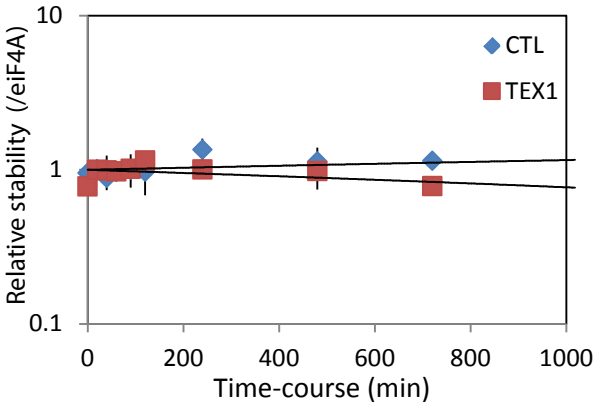
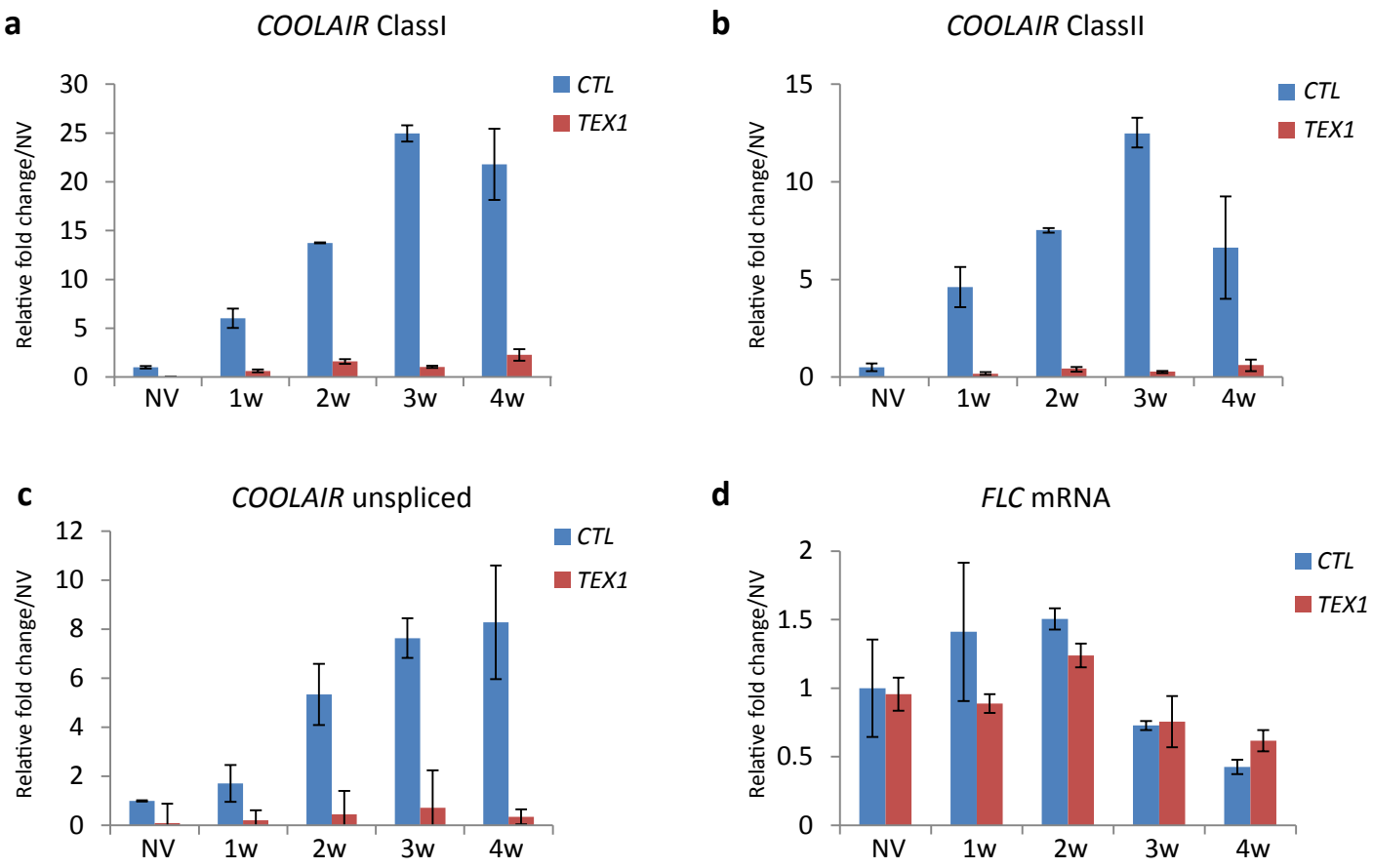


f

Stability elements in FLC 3'utr (last exon in capital, Stop codon in capital bold, 3'utr in small letters, stability hexamers in capital, italics, underlined):

ATGGA*GAATAA*TCATCATGTGGGAGCAGAAGC TG AGATGGAGAT GTCACCTGCTGGACAAATCTCCGA CAATCTTCCGGTGACTCTCCCACTACTTAAT**TAG** ccaccttaaatcggcggttg aaatcaaaatccaaaacatatataat tatgaagaaaaaaa*AAATAAG*atatgtaattattccgctgataag ggcgagcgtttgta tatcttaatactc*TCTCTT*tggccaa gagac tt*TGTGTG*tgatacttaagtagacggaactaagtcaatactatctg ttttaagacaaaaggttgatgaactttgtaccttattcgtgtgag

Fig. S1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COOLAIR ClassI
Relative fold change/NV
30
25
20
15
10
5
0
NV
1w
2w
3w
4w
CTL
TEX1
b
COOLAIR ClassII
Relative fold change/NV
15
10
5
0
NV
1w
2w
3w
4w
CTL
TEX1
c
COOLAIR unspliced
Relative fold change/NV
12
10
8
6
4
2
0
NV
1w
2w
3w
4w
CTL
TEX1
d
FLC mRNA
Relative fold change/NV
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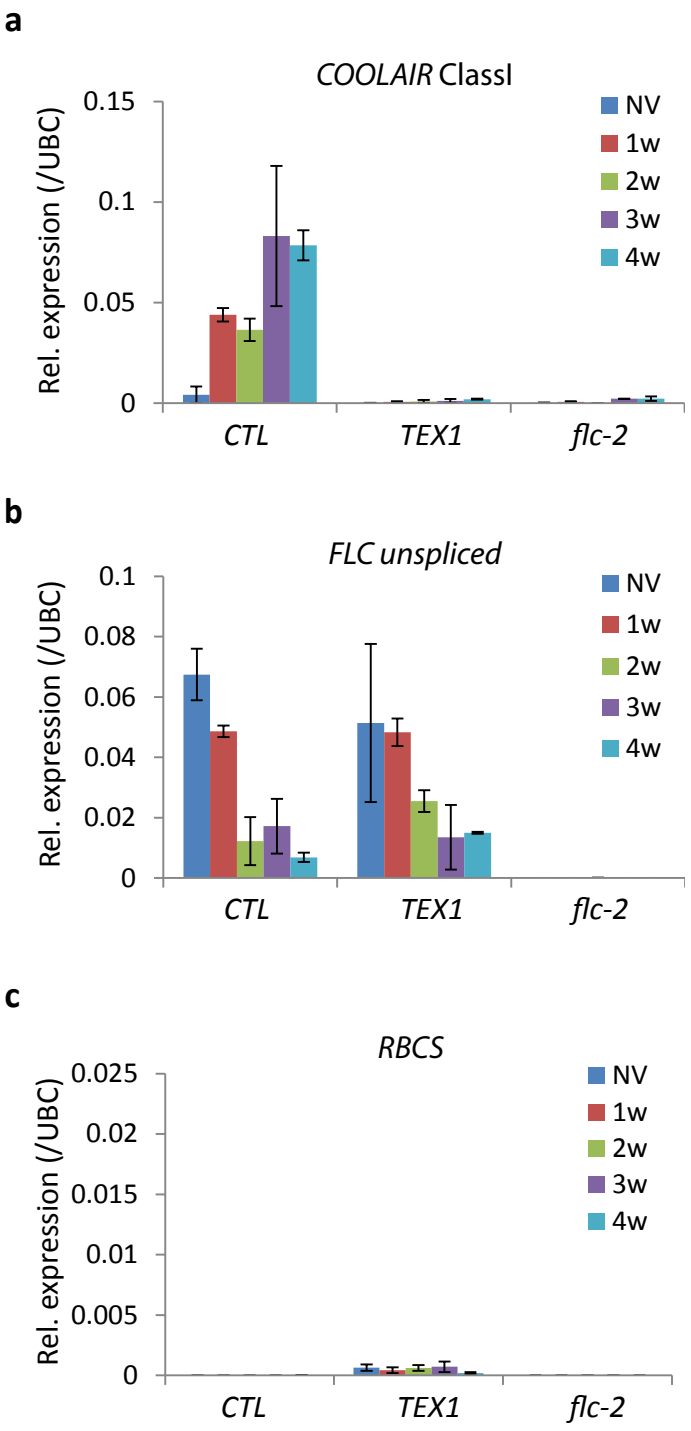
1.5
1
0.5
0
NV
1w
2w
3w
4w
CTL
TEX1

Fig. S2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Rel. expression (/UBC)
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0.1
0.05
0
NV
1w
2w
3w
4w
CTL
TEX1
flc-2
b
FLC unspliced
Rel. expression (/UBC)
0.1
0.08
0.06
0.04
0.02
0
NV
1w
2w
3w
4w
CTL
TEX1
flc-2
c
RBCS
Rel. expression (/UBC)
0.025
0.02
0.015
0.01
0.005
0
NV
1w
2w
3w
4w
CTL
TEX1
flc-2

Fig. S3

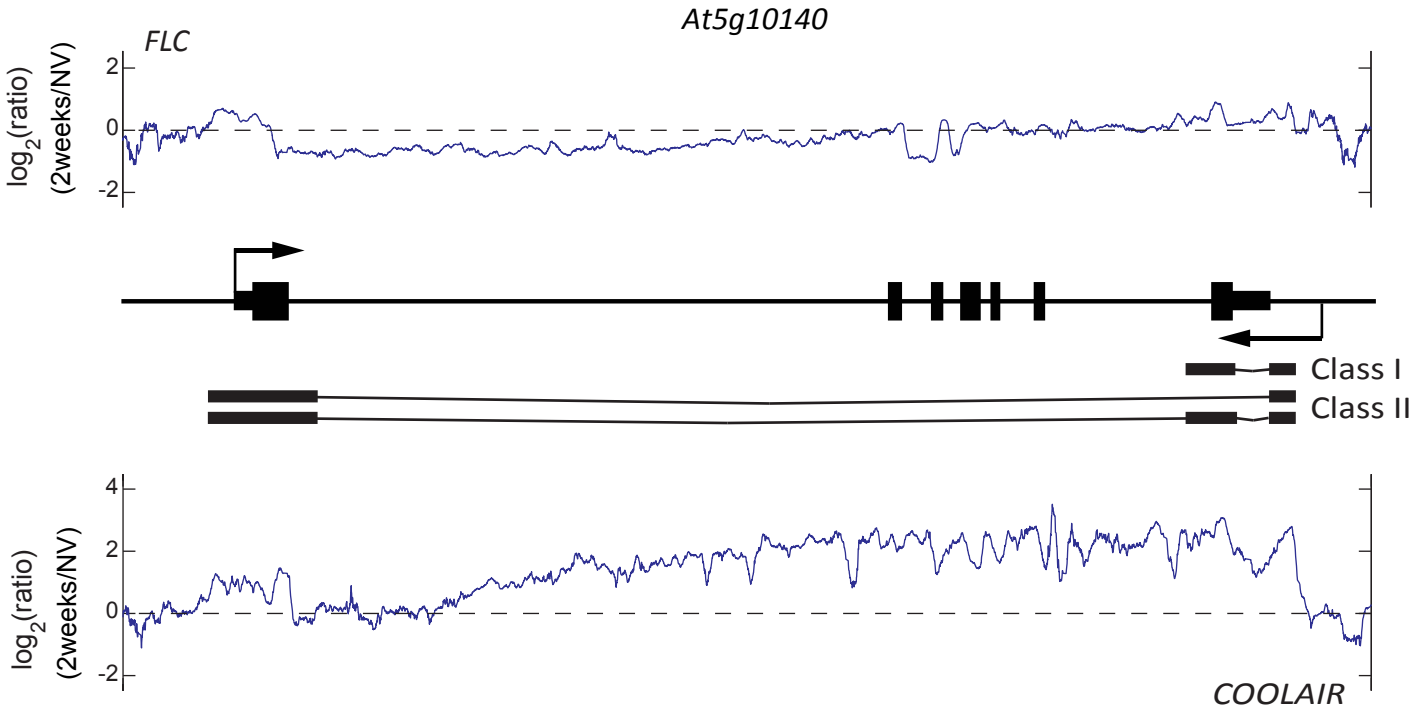
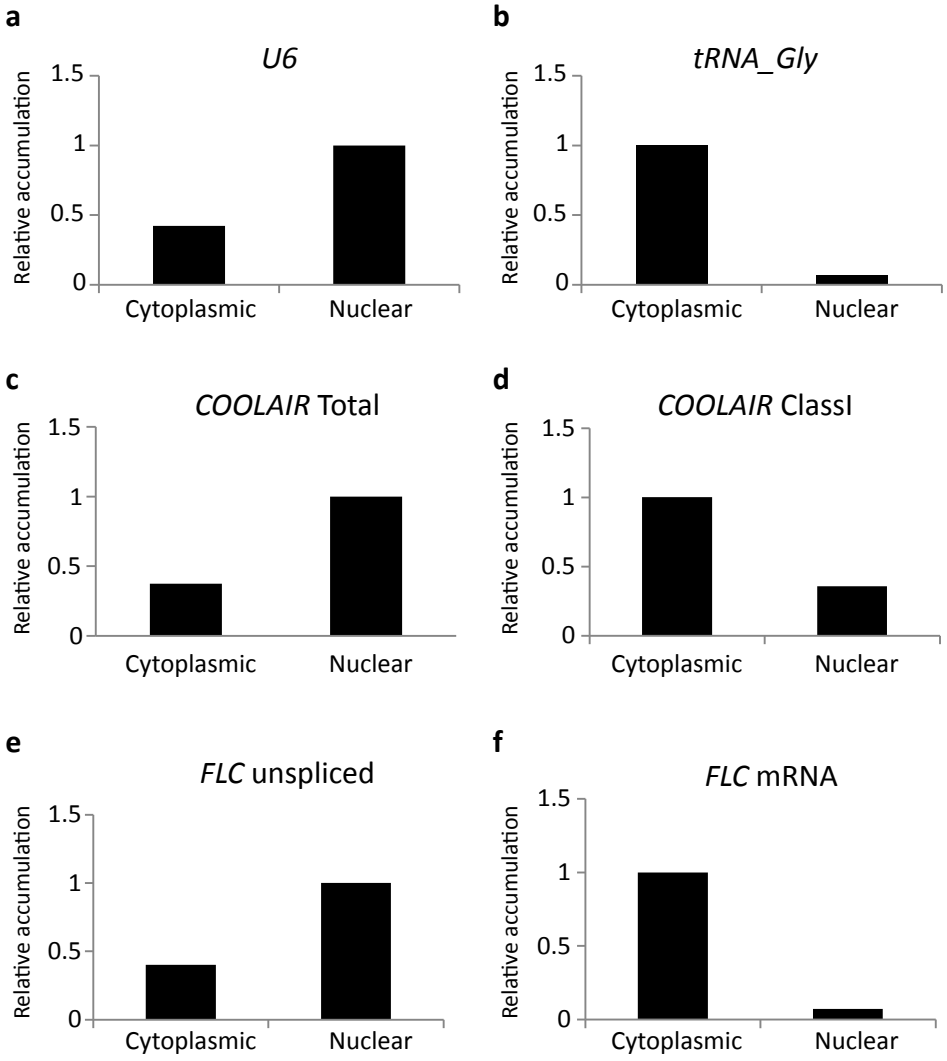

At5g10140
FLC
log2(ratio)
(2weeks/NV)
2
0
-2
Class I
Class II
4
2
0
-2
COOLAIR

Fig. S4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U6
b
tRNA_Gly
c
COOLAIR Total
d
COOLAIR ClassI
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FLC mRNA
Relative accumulation
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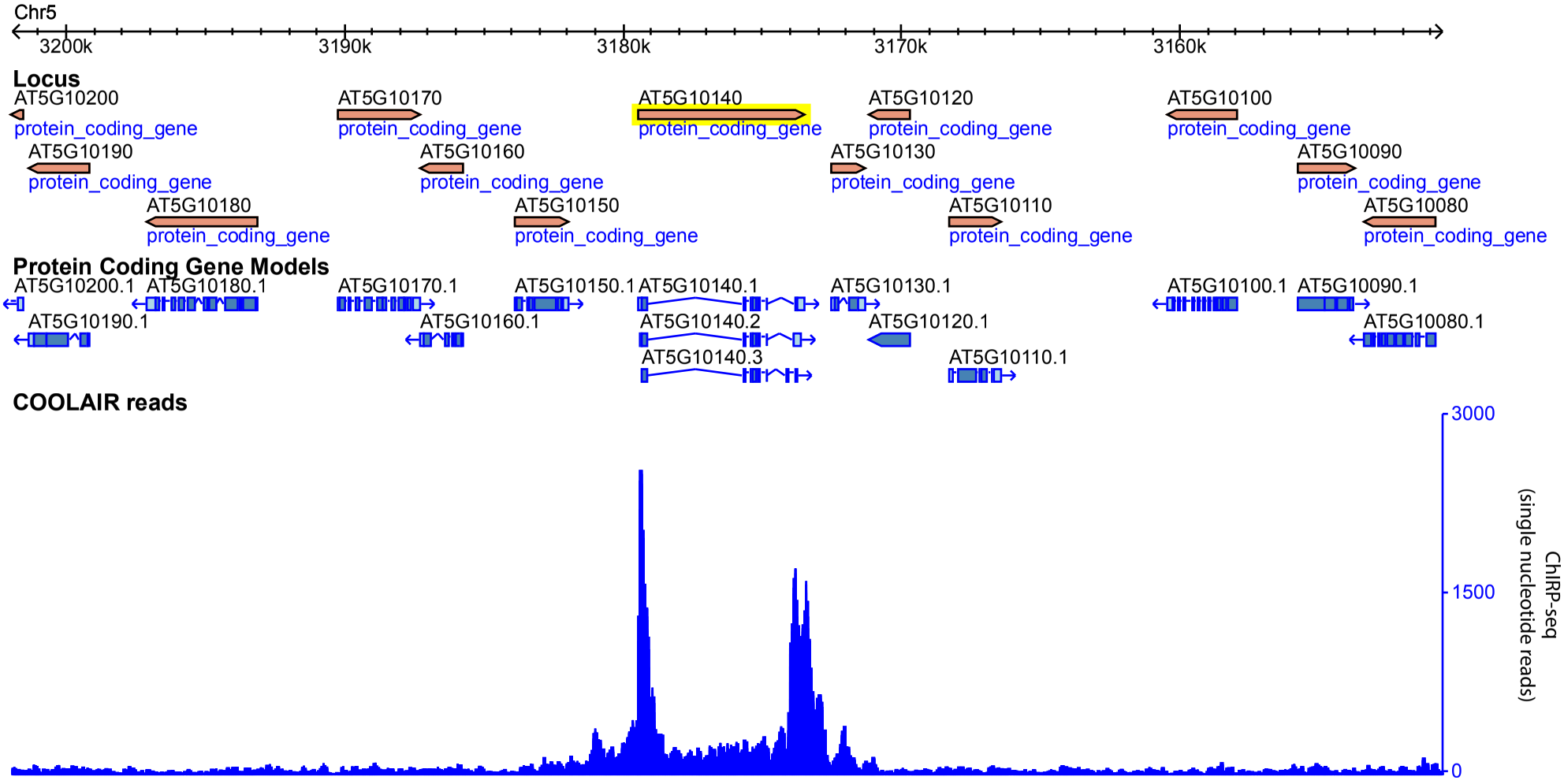
1
0.5
0
Cytoplasmic
Nuclear

Fig. S5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Locus
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protein_coding_gene
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protein_coding_gene
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Protein Coding Gene Models
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AT5G10160.1
AT5G10140.2
AT5G10120.1
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COOLAIR reads
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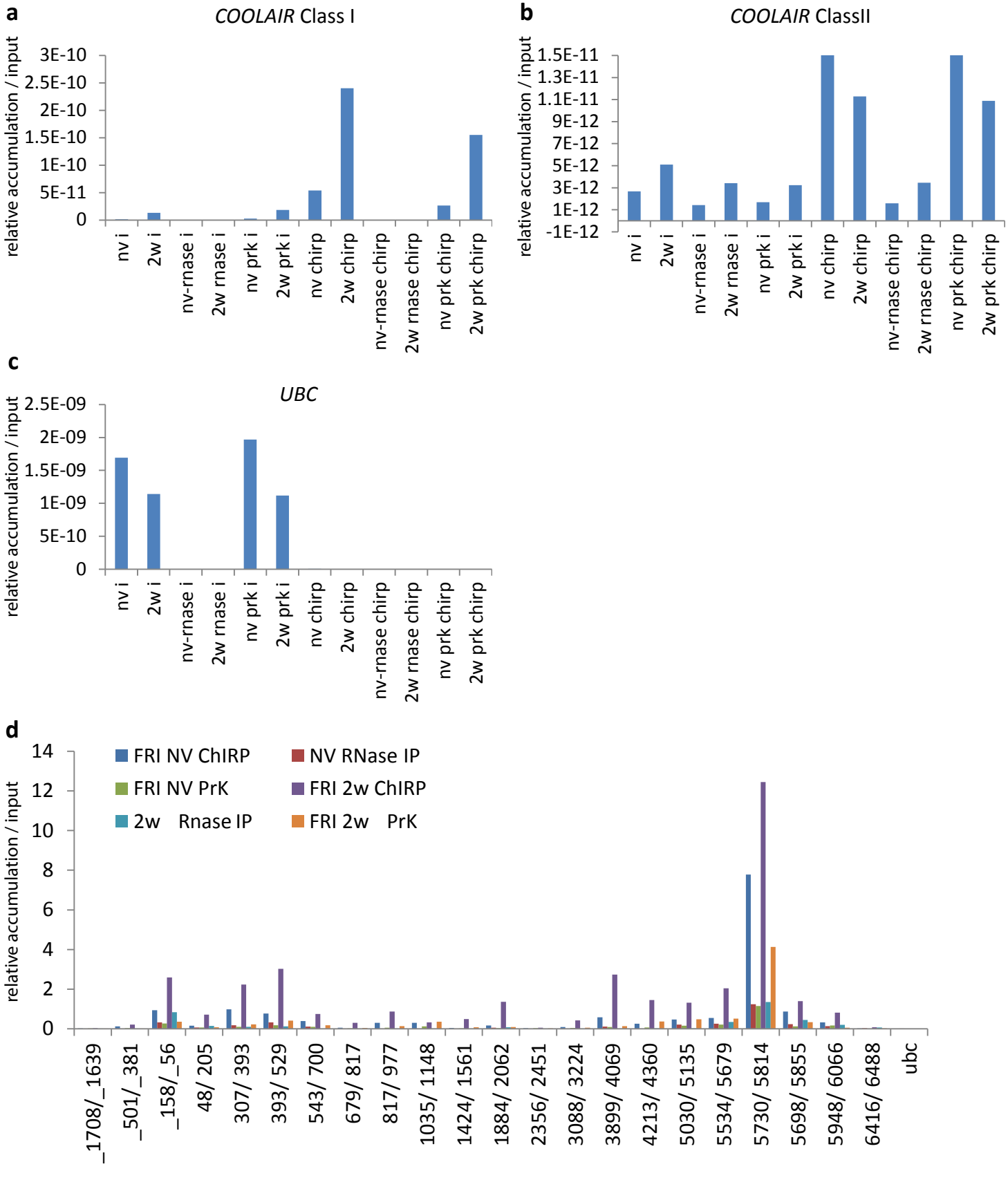
1500
0
ChIRP-seq
(single nucleotide reads)

Fig. S6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COOLAIR Class I
relative accumulation / input
3E-10
2.5E-10
2E-10
1.5E-10
1E-10
5E-11
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nv i
2w i
nv-rnase i
2w rnase i
nv prk i
2w prk i
nv chirp
2w chirp
nv-rnase chirp
2w rnase chirp
nv prk chirp
2w prk chirp
b
COOLAIR ClassII
relative accumulation / input
1.5E-11
1.3E-11
1.1E-11
9E-12
7E-12
5E-12
3E-12
1E-12
-1E-12
nv i
2w i
nv-rnase i
2w rnase i
nv prk i
2w prk i
nv chirp
2w chirp
nv-rnase chirp
2w rnase chirp
nv prk chirp
2w prk chirp
c
UBC
relative accumulation / input
2.5E-09
2E-09
1.5E-09
1E-09
5E-10
0
nv i
2w i
nv-rnase i
2w rnase i
nv prk i
2w prk i
nv chirp
2w chirp
nv-rnase chirp
2w rnase chirp
nv prk chirp
2w prk chirp
d
relative accumulation / input
14
12
10
8
6
4
2
0
FRI NV ChIRP
NV RNase IP
FRI NV PrK
FRI 2w ChIRP
2w Rnase IP
FRI 2w PrK
_1708/_1639
_501/_381
_158/_56
48/ 205
307/ 393
393/ 529
543/ 700
679/ 817
817/ 977
1035/ 1148
1424/ 1561
1884/ 2062
2356/ 2451
3088/ 3224
3899/ 4069
4213/ 4360
5030/ 5135
5534/ 5679
5730/ 5814
5698/ 5855
5948/ 6066
6416/ 6488
ubc

Fig. S7

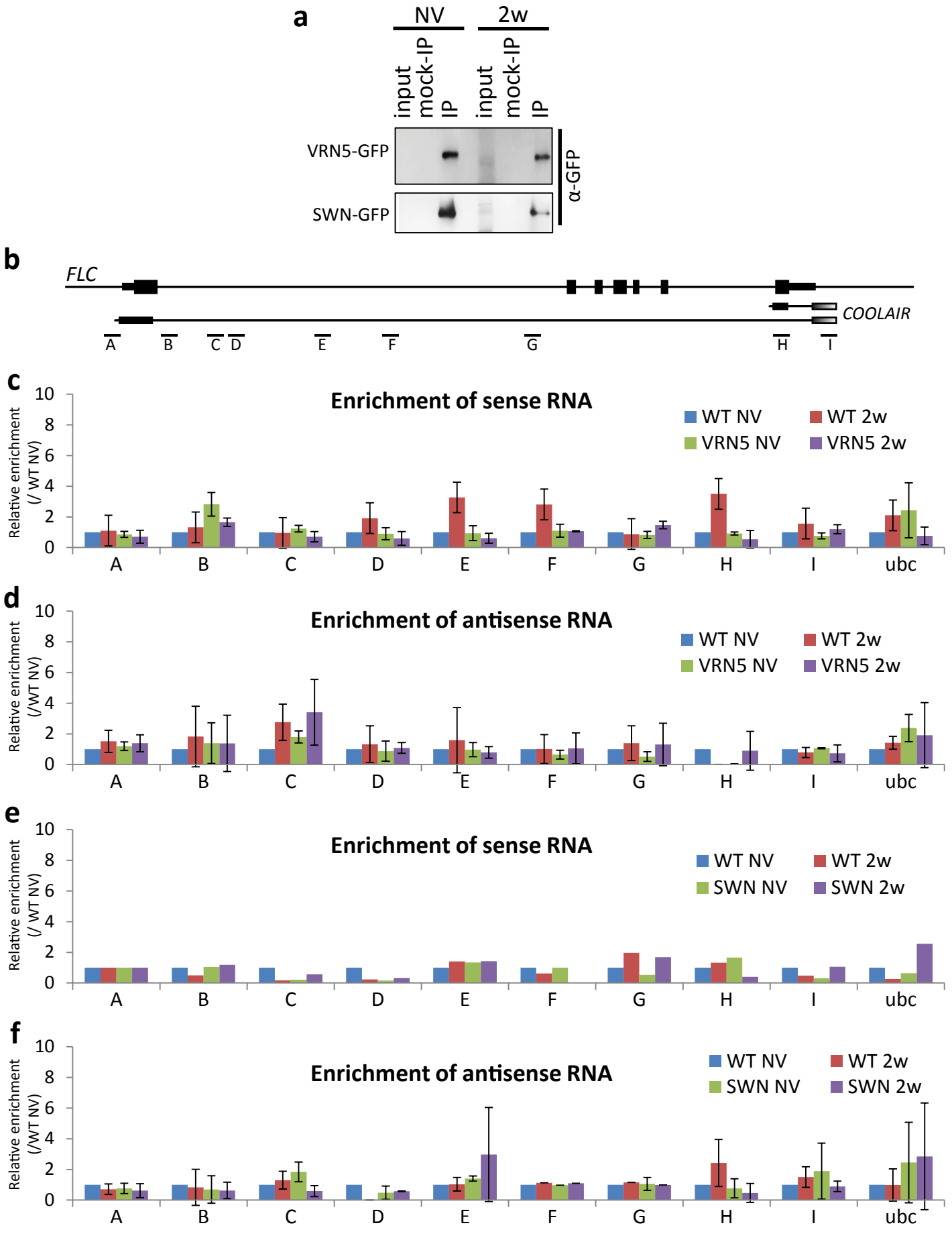

a
NV
2w
input
mock-IP
IP
input
mock-IP
IP
VRN5-GFP
SWN-GFP
α-GFP
b
FLC
COOLAIR
A
B
C
D
E
F
G
H
I
c
Enrichment of sense RNA
Relative enrichment (/ WT NV)
WT NV
WT 2w
VRN5 NV
VRN5 2w
A
B
C
D
E
F
G
H
I
ubc
d
Enrichment of antisense RNA
Relative enrichment (/WT NV)
WT NV
WT 2w
VRN5 NV
VRN5 2w
e
Enrichment of sense RNA
Relative enrichment (/ WT NV)
WT NV
WT 2w
SWN NV
SWN 2w
f
Enrichment of antisense RNA
Relative enrichment (/WT NV)
WT NV
WT 2w
SWN NV
SWN 2w

Fig. S8

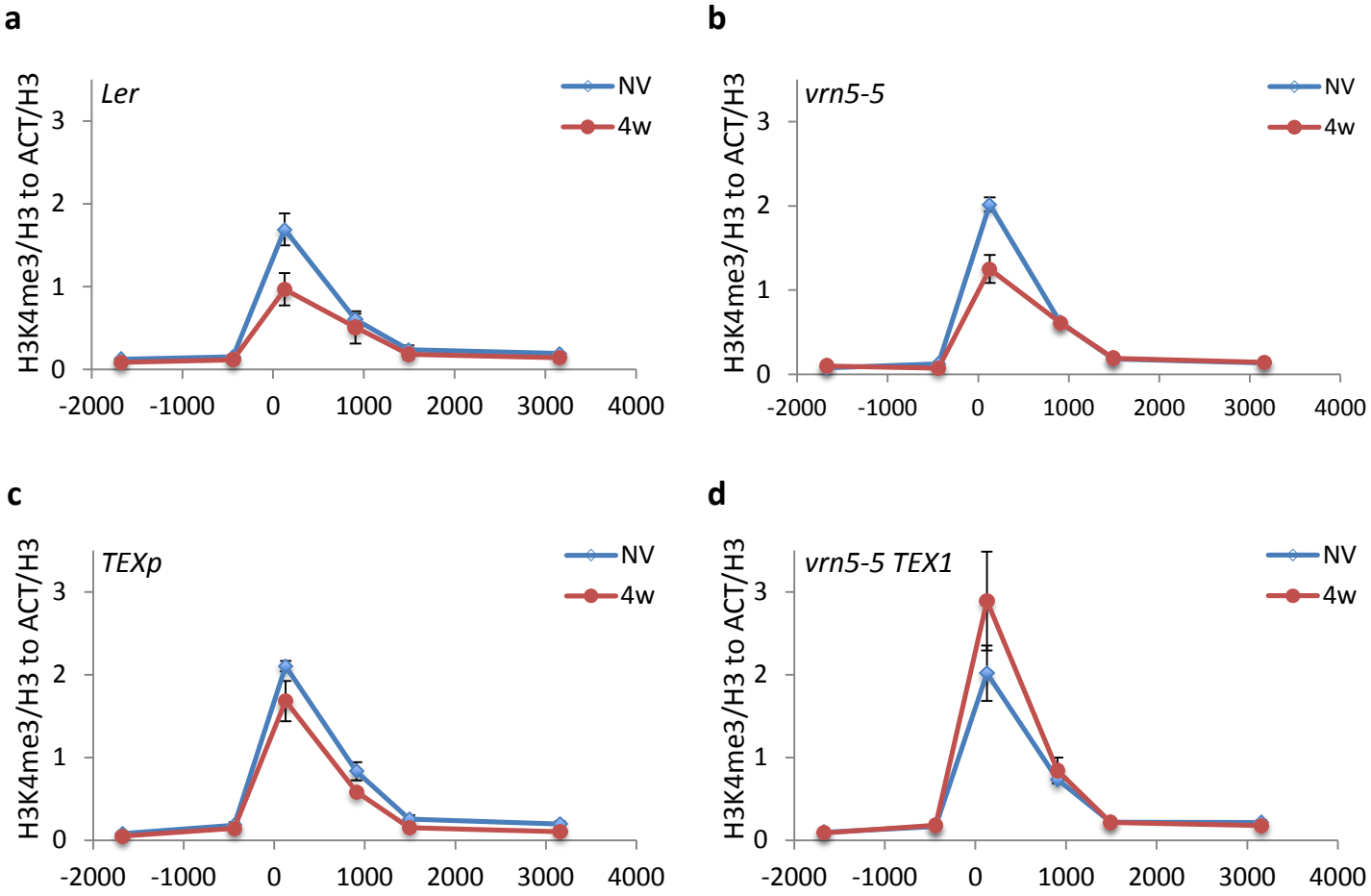
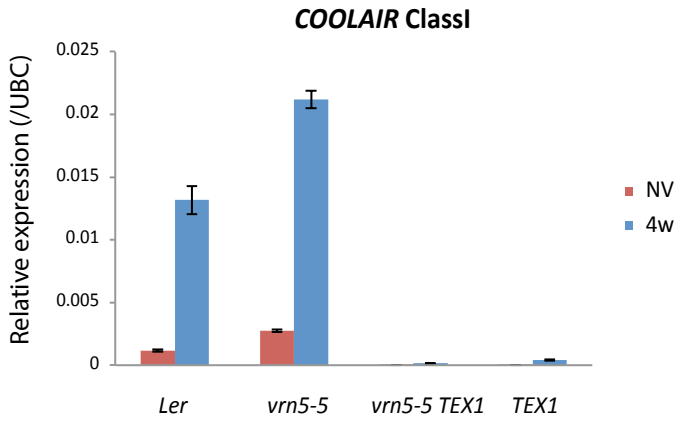
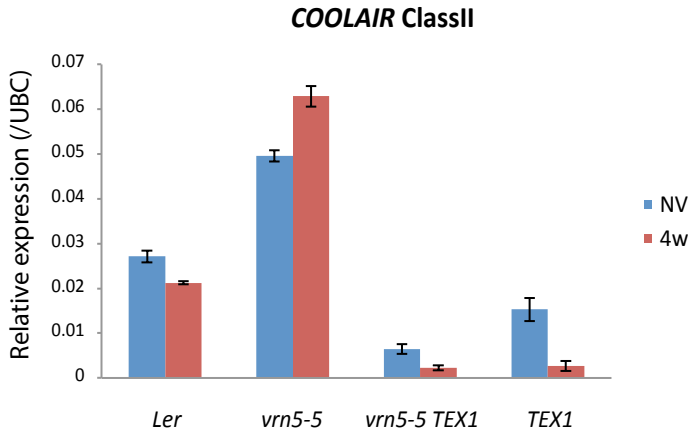

a
b
c
d
Ler
vrn5-5
TEXp
vrn5-5 TEX1
NV
4w
H3K4me3/H3 to ACT/H3
0
1
2
3
-2000
-1000
0
1000
2000
3000
4000

Fig. S9

a


COOLAIR ClassI
Relative expression (/UBC)
0.025
0.02
0.015
0.01
0.005
0
Ler
vrn5-5
vrn5-5 TEX1
TEX1
NV
4w

b


COOLAIR ClassII
Relative expression (/UBC)
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
Ler
vrn5-5
vrn5-5 TEX1
TEX1
NV
4w

c

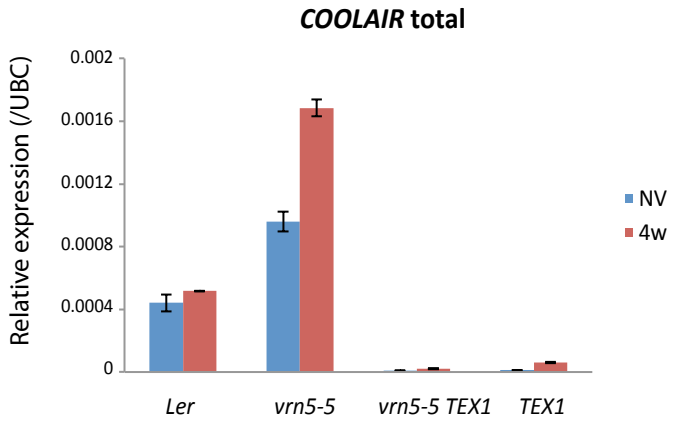

COOLAIR total
Relative expression (/UBC)
0.002
0.0016
0.0012
0.0008
0.0004
0
Ler
vrn5-5
vrn5-5 TEX1
TEX1
NV
4w

Fig.S10

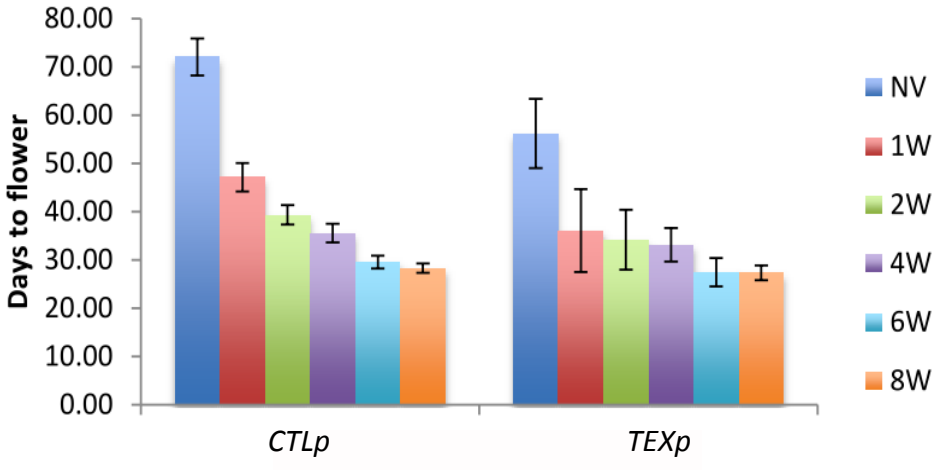

80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
Days to flower
CTLp
TEXp
NV
1W
2W
4W
6W
8W

Fig. S11