

clf mutation affects cold stress response and development in *Arabidopsis thaliana*

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Abstract

Background

Plants acquire freezing tolerance through cold acclimation, a period at low, non-freezing temperature during which gene expression is reprogrammed. This response is driven by the C-repeat binding factors (CBFs) but unfolds on chromatin shaped by the Polycomb Repressive Complex 2 (PRC2), which silences genes through the histone mark H3K27me3. Several Polycomb components converge on the *CBF* loci; what the histone methyltransferase CURLY LEAF (CLF) alone contributes to the cold response is not known.

Results

To ask whether CLF controls the cold response, we compared the transcriptomes of wild type and *clf-29* plants before cold, after three hours of cold, after four days of acclimation and after a further drop towards freezing temperature. The canonical pathway does not depend on CLF: the *CBF* genes and their *COR* targets were induced normally in the mutant. To see what the loss of CLF does instead, we compared the two genotypes within each condition. We found a broad misregulation that builds up with age at control temperature, from about one thousand to several thousand genes, and that acclimation almost abolishes, leaving fewer than two hundred. To establish whether any part of the cold response depends on CLF, directly or not, we tested the genotype-by-treatment interaction and scored the genes for H3K27me3. We found such a dependence almost only at acclimation, on genes that are depleted of H3K27me3 and relate to growth; the genes responding to short-term cold only in the mutant are enriched for H3K27me3 but not for the telobox motif that recruits PRC2. Finally, we measured survival to freezing: *clf-29* survived better than the WT, most clearly after acclimation.

Conclusions

CLF is not required for the canonical cold pathway, consistent with redundant Polycomb control of the *CBF* loci. Its loss mainly affects a developmental programme whose deregulation builds up during growth at control temperature and is strongly attenuated by cold. Cold acclimation thus emerges as a condition in which the H3K27me3-dependent effects of CLF can be separated from secondary, growth-related ones, and the improved survival of the mutant suggests that CLF restrains, rather than supports, the acquisition of freezing tolerance.

Background

Low temperature limits plant growth and distribution (1). In temperate species such as *Arabidopsis thaliana*, freezing tolerance is largely acquired through cold acclimation, whereby prior exposure to low, non-freezing temperatures protects the plant against a subsequent freezing event (2–4). Acclimation

rests on an extensive transcriptional reprogramming whose best characterised branch is the ICE1-CBF/DREB1 (INDUCER OF CBF EXPRESSION-C-repeat/DEHYDRATION-RESPONSIVE ELEMENT BINDING FACTOR) module (5, 6): upon cold perception, *CBF1-CBF3* are rapidly and transiently induced, and these transcription factors bind the *CRT/DRE* elements of *COR* (*COLD-REGULATED*) genes, activating hundreds of them within hours (7–9). Constitutive expression of a single *CBF* gene is sufficient to increase freezing tolerance and to reconstitute much of the *COR* regulon, establishing the CBF module as a master switch of acclimation (10–12). Within this regulon the three *CBFs* are not equivalent - *CBF2* negatively regulates *CBF1* and *CBF3* (13), defining distinct gene classes (14) - and the pathway is best viewed as a structured gene-regulatory network rather than a single linear branch (15). This switch is not a linear cold-to-*CBF* cascade: it is gated by both light and development through interactions between the CBF and the PIF (PHYTOCHROME-INTERACTING FACTOR) factors, and photoperiodic control of *CBF* expression (16–18). In addition, the *CBF* cluster is itself subject to fine-tuning by non-coding transcription from the *CBF1-CBF3* intergenic region: read-through of the cold-induced lncRNA *SVALK*A produces an antisense transcript that limits *CBF1* expression (19), whereas the antisense RNA *CAS* forms an R-loop that facilitates the transcription of *CBF1* and *CBF3* (20).

Superimposed on this transcription factor network is a layer of chromatin regulation that sets the accessibility and the heritable expressive state of stress-responsive genes. Epigenetic mechanisms are widely involved in the response of plants to abiotic stresses, from DNA methylation to histone modifications, to ncRNAs (21–24). Polycomb Repressive Complex 2 (PRC2) lays down H3K27me₃, a repressive mark silencing thousands of developmental and stress-responsive loci (25, 26). This mark is antagonised by Trithorax-group activities that deposit the active mark H3K4me₃ (27, 28). In *Arabidopsis* the PRC2 catalytic core is provided by three partially redundant E(z)-homolog methyltransferases - CLF (CURLY LEAF), SWN (SWINGER) and the seed-specific enzyme MEA (MEDEA) (29, 30) - with CLF being the predominant activity in vegetative tissue and SWN displaying a partially redundant function (31). A central and only partly resolved question is how PRC2 is targeted to specific genes: short telomere-repeat-like motifs (teloboxes, AAACCCT) (32) act as *cis*-recruitment elements, and TRB (TELOMERE-REPEAT-BINDING) proteins bridge these motifs to CLF/SWN-PRC2 to direct H3K27me₃ deposition (33).

A growing body of work places chromatin regulation at the heart of the cold response but also thickens the simplest model. During prolonged cold (vernalisation), quantitative and memory-like silencing of the floral repressor *FLC* is achieved through opposing H3K27me₃ and H3K36me₃/H3K4me₃ dynamics, a paradigm for converting a temperature signal into a stable chromatin state (34, 35). Many *COR* genes carry H3K27me₃ in the absence of cold (36). The mark might be expected to keep them silenced until cold triggers its removal; yet at *COR15A* and *ATGOLS3* the mark decreases only during cold exposure, after transcription has started (36). Indeed, more recently, genome-wide profiling during short-term cold induction showed that H3K4me₃ and H3K27me₃ redistribute rapidly but on different gene sets, and that in the *clf* mutant many cold-regulated genes, although deprived of the H3K27me₃ mark as expected, do not show an altered induction profile (37). This suggests that H3K27me₃ does not simply prevent the induction of *COR* genes but rather may be involved in modulating the amplitude of the induction during long-term or de-acclimation responses or only be fully unmasked in *clf* *swn* double mutants (37, 38). On

the other hand, targeted studies show that Polycomb factors do shape specific cold-relevant loci: the PRC2-associated VIL1 (39) (VERNALIZATION INSENSITIVE 3-LIKE 1) establishes a repressive, H3K27me₃-marked chromatin environment over the tandem *CBF* cluster and, together with *CBF1*, controls growth retardation at low ambient temperature by regulating chromatin looping and accessibility (40). Beside this, the cold-inducible lncRNA *SVALKa* recruits PRC2 to the coding region of *CBF3*, where H3K27me₃ deposition silences *CBF3* and shapes the timing of its decline after induction; a *SVALKa*–PRC2 module that couples non-coding transcription and Polycomb repression directly to a core cold transcription factor (41). Together these findings indicate that the cold-acclimation transcriptome is shaped not only by the CBF cascade but also by Polycomb-based repression and its context-dependent release. However, the specific set of genes under CLF control during acclimation, as well as the developmental stage at which this control operates, and the *cis*-mechanism of their targeting remain incompletely defined.

To address these questions we performed an RNA-seq experiment comparing wild type (WT) and the *clf* (*clf-29*) mutant across a controlled time course spanning cold induction, cold acclimation, and freezing induction with respect to untreated control, applied in both acclimated and non-acclimated backgrounds. The design of our RNA-seq asks not only which genes differ between WT and *clf-29*, but whether this difference itself changes with the cold treatment. We then investigate the nature of these genes: (i) whether they carry H3K27me₃ in the WT background (37), (ii) whether their promoters contain the telobox motifs that recruit PRC2 (33), and (iii) whether and how the Polycomb machinery and the CBF module themselves crosstalk in the cold response (40). Our results demonstrated that, across the time course, lack of CLF counted most before cold. In non-acclimated plants thousands of genes were misregulated in *clf-29*, about half of them expressed at a higher level than in the WT, i.e. the fraction expected to depend directly on CLF as a repressor. Four days at 4°C almost counteracted this difference: after acclimation the two genotypes were nearly indistinguishable. This compensation did not extend to the canonical cold pathway, which responded normally in the mutant, although *clf-29* plants survived freezing better than the WT after acclimation. Canonical Polycomb targets are known to carry the telobox motifs bound by the PRC2-associated TRB proteins (33), whereas the *clf*-specific cold sets are enriched in H3K27me₃ but show no such signature, pointing to a recruitment route other than the telobox–TRB axis.

Methods

Plant material and growth conditions

Arabidopsis thaliana Columbia-0 (Col-0) was used as WT. The *clf-29* loss-of-function line, which lacks the CLF histone methyltransferase catalytic activity, was used for the transcriptomic analysis and for the freezing assays. Seeds were surface-sterilised and sown on filter paper laid on half-strength Murashige and Skoog agar (0.8% agar, pH 5.7), then stratified for three days at 4°C in the dark. After germination, seedlings were transferred, together with the filter paper, into SacO2 microboxes and grown at 22°C under long-day conditions (16 h light / 8 h dark, cool-white fluorescent light, 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$). All

samples for the transcriptomic analysis were grown in vitro under these conditions at CREA (Rome, Italy). For the freezing assays, seeds were germinated on half-strength Murashige and Skoog agar plates and the seedlings were then transplanted into soil and grown for two weeks under the same temperature and photoperiod regime at CRAG (Barcelona, Spain). During the acclimation treatment the light intensity was reduced to $100 \mu\text{mol m}^{-2} \text{s}^{-1}$.

Cold acclimation, freezing treatment and phenotypic scoring

The freezing set-up was adapted from Perea-Resa et al. (42). Plants were grown at 22°C under long-day conditions for 14 days and then assigned to one of three temperature regimes: No Cold (NC), maintained at 22°C throughout and providing the growth control; Acclimation (AC), 4°C for 4 days; and No Acclimation (NA), kept at 22°C for the same 4 days. Acclimated and non-acclimated plants were then exposed to freezing at -7°C for 6 hours. The identical freezing temperature was applied to both, which makes the non-acclimated treatment severe. All plants were then returned to standard growth conditions for 7 days of recovery, after which survival was scored. Because an in vitro set-up does not reproduce soil conditions – and freezing injury in particular depends on the substrate – the experiments reported here were performed in soil, in the facilities of the Centre for Research in Agricultural Genomics (CRAG, Barcelona).

Survival was scored after the recovery period as the number of surviving plants out of the total plants in each pot, with 16 plants per pot and two to three pots per genotype and regime in each of three independent biological replicates, six to seven pots in total (three experimental rounds). Survival fractions were analysed by two-way analysis of variance with genotype and temperature regime as factors and their interaction; the pot was the unit of analysis and the biological replicate was not modelled as a factor. In addition, survival per pot was compared between genotypes within each regime by a Welch's t test (Fig. 6g). No replicate was excluded from any analysis. Tests were computed with statsmodels v0.14.6 (43) and SciPy v1.17.1 (44).

RNA-seq experimental design

WT and *clf-29* seedlings were sampled across a cold time course. Fourteen day-old seedlings were collected as the No Cold control (NC) or after 3 h at 4°C as the Cold Induced time point (CI). Seedlings held for 4 days at 4°C were collected as the Acclimation time point (AC), or subjected to a second temperature decrease (3 h, 1°C/h , in the dark) and collected as acclimated-plus-freezing (ACFr). Plants kept for the same 4 days at 22°C were collected as the No Acclimation control (NAC) or transferred to 4°C to reach 1°C over 3 h in the dark and collected as non-acclimated-plus-freezing (NACFr). All conditions were sampled in three biological replicates, giving the 36 libraries analysed here.

RNA extraction, library preparation and sequencing

Total RNA was extracted from whole seedlings with the Direct-zol RNA MiniPrep Kit (Zymo Research, Irvine, CA, USA), homogenising the tissue in Direct-zol reagent and following the manufacturer's protocol; contaminating genomic DNA was removed by on-column DNase I digestion. RNA concentration was measured with the Qubit RNA High Sensitivity (HS) Assay Kit on a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), and RNA Integrity Numbers were determined on a LabChip GX Touch HT Nucleic Acid Analyzer using a DNA 5K/RNA/CZE chip with the Pico RNA Assay Reagent kit (PerkinElmer, Waltham, MA, USA), with a mean RNA quality score of 8.4. Poly(A)-tailed RNAs were enriched from total RNA with Nextflex PolyA Beads 2.0 (PerkinElmer), and libraries were prepared from 500 ng of total RNA with the Nextflex Rapid Directional RNA-Seq Kit 2.0 and Nextflex Unique Dual Index Barcodes (PerkinElmer), with eight PCR cycles. Library quantity was measured with the Quant-iT 1X dsDNA High Sensitivity Assay Kit on a FLUOstar Omega plate reader (BMG LABTECH, Ortenberg, Germany), and fragment size distribution by capillary electrophoresis on a LabChip GX Touch Nucleic Acid Analyzer with an XMark HT chip and the DNA NGS 3K Assay kit (PerkinElmer). Pooled libraries were diluted to 350 pM and sequenced on an Illumina NovaSeq 6000 with a NovaSeq 6000 S4 Reagent Kit v1.5 (300 cycles) (Illumina, San Diego, CA, USA), generating 2 x 151 bp paired-end reads, with approximately 18.5 million read pairs per sample. Basecalling and demultiplexing were performed with NovaSeq Control Software (Illumina).

RNA-seq data analysis

RNA-seq reads were preprocessed using the FASTQ Toolkit (45), with parameters `--adapter-trimming-stringency 0.90 --min-length 100 --min-quality-mean 30 --trim-qual-5 30 --trim-qual-3 30 --adapters-3 AGATCGGAAGAGCACACGTCTGAACTCCAGTCA,AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT --filter-non-pf --filter-singleton`. Salmon (46) v1.3.0 was employed to quantify transcript-level abundance, using a full decoy transcriptome index created using the *Arabidopsis thaliana* cDNA and ncRNA transcriptomes and the TAIR10 genome, all downloaded from Ensembl (47) Plants release 56. Quantification was performed using the following parameters: `--gcBias --numBootstraps 30 -l ISR --validateMappings`. Gene-level estimated counts were obtained from the Salmon quantification files using the Tximport (48) v1.18.0 R package and rounded to the nearest integer. Gene symbols were mapped to TAIR identifiers with the `org.At.tair.db v3.22.0` R package (49).

DESeq2 v1.50.2 R package was used to perform count-based differential expression analysis. Genes reaching at least 10 raw counts in at least three samples were retained, giving 21,285 of the 32,311 annotated genes, and this set was used as the expression universe for all downstream analyses. Normalised counts were variance-stabilised (VST) for PCA and clustering analyses. PCA was performed using the PCA function from the scikit-learn (50) v1.9.0 Python package on the variance-stabilised values of all 21,285 retained genes, obtained with the `vst` function from the DESeq2 v1.50.2 R package, run in blind mode, centered by gene and without per-gene scaling. The percentage of variance explained by each component and the correlation of components with design variables (acclimation, cold, age, genotype) were computed to interpret the axes, using the `pearsonr` function from the SciPy v1.17.1

Python package; for these correlations, samples were coded as cold-exposed when they had experienced low temperature at any point of the time course, including acclimated samples. For clustering, counts were variance-stabilised with the `vst` function from the same package, fitted on the experimental design; the 4,000 most variable genes were selected using the `rowVars` function from the `matrixStats` v1.5.0 R package on those values. Clustering was performed on the per-gene z-scores of the selected genes, capped at ± 4 , using the `hclust` function from the `stats` R package of R v4.5.3 on Euclidean distances with complete linkage, and ten clusters were obtained with the `cutree` function ($k = 10$); cluster membership of every gene is provided in Table S1. The `matplotlib` v3.11.0 (51) Python package was used to generate the gene expression heatmap based on the 4,000 most variable genes, following Z-score normalization of their VST-transformed expression values, and the per-cluster mean z-score profiles.

Differential expression between *clf* and WT were assessed for each condition using the `results` function from the `DESeq2` v1.50.2 R package. Genes were considered differentially expressed (DEG) based at adjusted $p < 0.05$ (Benjamini-Hochberg correction) (52), with no fold change threshold applied. DEGs were classified as up- or down-regulated according to the sign of the log2 fold change.

In addition, differential expression was assessed separately for each genotype by comparing each cold treatment with its corresponding non-cold control: CI versus NC, AC versus NAc, NAcFr versus NAc, and ACFr versus AC. For each of these comparisons, the genes detected as differentially expressed in *clf* but not in WT were classified as *clf*-specific cold-responsive genes.

The genotype $\times$ condition interaction was tested in `DESeq2`. A likelihood-ratio test comparing the full model (genotype, condition and their interaction) with the reduced model lacking the interaction term identified genes whose response differs between genotypes anywhere in the design (2,064 genes at adjusted $p < 0.05$). The interaction was then tested separately at each cold transition as a Wald test on the corresponding interaction contrast; the counts reported in the text and in Fig. 6a are those per transition tests (adjusted $p < 0.05$).

Transcription-factor family analysis

Transcription factors and their family assignment were taken from the Arabidopsis transcription-factor list of PlantTFDB (53), which contains 1,717 factors in 58 families; the few genes annotated to more than one family are counted in each of them. Enrichment of each family among the CLF-dependent transcription factors was tested against the same 1,717-factor universe using the one-sided `fisher_exact` function (`alternative = 'greater'`) from the `SciPy` v1.17.1 Python package, with Benjamini-Hochberg correction across families. In Fig. 3g only families present in at least five conditions or with at least fourteen differentially expressed members are shown.

Gene Ontology enrichment analysis

GO Biological Process over-representation was computed using the `enrichGO` function from the `clusterProfiler` (54) v4.18.4 R package against the `org.At.tair.db` v3.22.0 annotation (`GO.db` v3.22.0, `AnnotationDbi` v1.72.0), using the expressed 21,285-gene set as universe, Benjamini-Hochberg correction and a significance cutoff of adjusted $p < 0.05$; for the *clf*-specific sets only terms with adjusted $p < 0.01$ are displayed (Fig. 4g-j). Redundant terms were collapsed using the `simplify` function from the same package, with Wang semantic similarity computed through the `GOSemSim` v2.36.0 R package (55) and a similarity cutoff of 0.7, retaining the term with the smallest adjusted p-value in each group.

Regulatory networks inference

Co-expression networks were inferred using the `GENIE3` v1.32.0 R package (56), with default tree parameters, separately for each cold condition, using the *clf*-specific transcription factors identified under that condition as candidate regulators and the corresponding *clf*-specific genes as targets. Networks were inferred from the 12 samples included in each comparison. The top 1% of edges by weight was retained, and the 10 regulators with the largest summed edge weight within that top 1% are shown. Given the limited number of samples underlying each network, these edges should be interpreted as putative co-regulatory relationships rather than experimentally validated interactions. Repeating the inference with ten random seeds recovered the five highest-ranking regulators of each condition in all ten runs.

Analysis of H3K27me3-marked genes

An H3K27me3 ChIP-seq map of WT tissue was used (37); GEO accession GSE255445. The dataset comprises three time points — naive, 3 h and 3 d at 4°C — each with two biological replicates; the enrichment in Fig. 4o was computed on the peaks reproducible between the two replicates of the 3 d stage (5,879 regions); the corresponding sets at the naive and 3 h stages comprise 6,007 and 5,724 regions and give the same enrichment for the CI set (odds ratio 1.84 and 1.97). A gene was scored as H3K27me3-marked if its lowest annotated coordinate, used as a strand-agnostic proxy for the transcription start site, fell within a peak; gene coordinates were taken from `org.At.tair.db` v3.22.0 and peak-to-gene assignment was computed with custom interval code using the `pandas` v2.3.3 and `NumPy` v2.4.6 (57) Python packages. Enrichment of each gene set among H3K27me3-marked genes was assessed using the two-sided `fisher_exact` function from the `SciPy` v1.17.1 Python package against a common background universe, with Benjamini-Hochberg correction across the sets tested in each analysis (Fig. 4o, Fig. 6b, Fig. 6e and Fig. S5d). Of the 21,285 genes retained after expression filtering, 20,689 had genomic coordinates in the annotation and were therefore included in the analysis. The remaining 596 genes, comprising recently annotated non-coding loci and organellar genes, could not be assessed for H3K27me3 marking because they lacked suitable TSS coordinates and were excluded from the background. Among the 20,689 genes in the common universe, 2,266 (11.0%) were H3K27me3-marked. This same universe was used for all enrichment analyses to ensure comparability of

percentages and odds ratios. As ChIP-seq data were not available for the mutant, all inferences on the mark are indirect.

Cis -motif (telobox/TRB) analysis

Promoter sequences (1,000 bp upstream of the transcription start site, strand-aware) were retrieved from the TAIR10 genome (Ensembl Plants release 56) through the Ensembl Plants REST API for the target sets (686 promoters at CI, 2,571 at AC) and for a background set of 3,000 expressed genes drawn at random. Motif occurrences were identified with FIMO (58) from the MEME Suite (59) v5.5.9 against the JASPAR 2024 plant CORE non-redundant matrices ($p < 1 \times 10^{-4}$). For each motif, the number of promoters containing at least one occurrence in the target set versus the background was compared by one-sided Fisher's exact test (fisher_exact function with alternative='greater', SciPy v1.17.1 Python package), with Benjamini-Hochberg correction across the matrices tested in each set (702 for the CI set, 703 for the AC and the interaction sets).

Definition of the cold-reversal sets

Two different thresholds were used to define the cold-reversal sets, one for each comparison. For the overlap between acclimation and freezing without acclimation, a gene was scored as attenuated when its *clf*-versus-WT effect decreased by more than 0.3 log2 units relative to the non-acclimated control. For the comparison of the early-cold and the freezing routes, a gene was scored as attenuated when its effect was at least halved relative to the starting condition of that route. Overlaps between sets were tested using the fisher_exact function from the SciPy v1.17.1 Python package.

Difference-of-differences of the Polycomb machinery genes

For each chromatin regulator, the cold response of the mutant was compared with that of the WT as a difference of differences (DoD) on the variance-stabilised values, tested using the ttest_ind function from the SciPy v1.17.1 Python package with equal_var = True, that is a two-sample Student t-test, on the replicate-level differences.

RNA extraction, reverse transcription and RT-qPCR

Total RNA was quantified on a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and 1 µg of each sample was reverse transcribed with the PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa Bio), which includes a genomic DNA removal step. Quantitative PCR was performed with the SensiFAST SYBR No-ROX Kit on a QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific), on the same six sample types used for the transcriptome. Primer sequences are listed in Table S12. Two independent biological replicates were assayed with two to three technical replicates per sample.

Quantification, normalisation and statistical analysis of RT-qPCR data

Relative expression was calculated by the comparative $\Delta\Delta C_t$ method (60) with an assumed amplification efficiency of 2, using *ACTIN 2* (*ACT2*, AT3G18780) as the reference (61). The non-acclimated WT sample was used as the common calibrator for both genotypes, so that constitutive differences between genotypes and cold induction within each genotype are expressed on the same scale; normalising each genotype to its own non-acclimated sample would set both to unity and remove the constitutive difference that the comparison requires. Measurements were screened before analysis and excluded if the target C_t exceeded 30, at which quantification of a low-abundance transcript becomes unreliable, if the reference C_t exceeded that plate's median by more than two cycles, indicating insufficient cDNA, or if fewer than two technical wells amplified; a genotype comparison was retained only when both genotypes passed. Significance was assessed by Welch's t-test on the ΔC_t values of the technical replicates. Tests were computed with SciPy v1.17.1 and statsmodels v0.14.6 Python packages.

Claude (Anthropic) was used for language editing and for consistency and verification checks on already generated analyses; the authors take full responsibility for the content.

Results

Global transcriptomic dynamics during cold acclimation and freezing stress

To investigate how CLF activity influences gene expression dynamics during plant response to cold stress, we designed an RNA-seq time course experiment with three biological replicates per group (Fig. 1a). 14 day-old WT and *clf-29* seedlings were collected after 3 hours at 4°C to investigate early response to cold treatment (Cold Induction, CI), with respect to the untreated control samples (No Cold, NC). To analyse transcriptome changes associated with acclimation, 14 day-old seedlings were exposed to 4°C for 4 days and collected as Acclimation (AC) samples. In parallel, 18 day-old seedlings grown at 22°C were collected as a control for non-acclimated conditions (No Acclimation, NAc). To investigate transcriptional responses to freezing stress, acclimated seedlings were subjected to a temperature decrease from 4°C to 1°C for 3 hours (1°C/h) and collected as Acclimation and Freezing Induction (ACFr) samples. Non-acclimated seedlings exposed to the same freezing treatment were collected as No Acclimation and Freezing induction (NAcFr) samples. After retaining genes with at least 10 read counts in three samples, 21,285 out of 32,311 annotated genes were used for downstream analysis.

Principal component analysis (PCA) (Fig. 1b-d) showed that acclimation was the main source of variance in the dataset (Fig. 1b). Principal component 1 (PC1) separated acclimated from non-acclimated samples and explained 62.6% of the total variance; the acclimated samples (AC, ACFr, both in WT and *clf* background) correlated positively with PC1 (Fig. 1b, d). PC2 (10.2%) captured the response to cold, separating the non-cold controls (NC, NAc) from the cold- and freezing-treated samples (CI, NAcFr) (Fig. 1d). PC3 (7.7%) separated 14 from 18 day-old seedlings, reflecting seedling age and, with it, developmental differences (Fig. 1d). The *clf* mutation was associated with PC4 (4.4%), a minor but

coherent genotype axis. Along this component the clearest WT-versus-*clf* separation was observed at cold induction (*clf* CI, $|r| = 0.47$, $p < 0.01$; Fig. 1d).

Consistently, analysis of the 4,000 most variable genes revealed a marked reprogramming of gene expression associated with acclimation establishment (Fig. 2a). This major reprogramming overshadowed the expression differences due to the *clf-29* mutation during cold stress response. Indeed, differences between WT and *clf-29* were more evident under control conditions, particularly in 18 day-old seedlings, while cold and freezing treatments reduced these differences. Clustered expression profiles based on the 4,000 most variable genes (Table S1), together with the corresponding Gene Ontology (GO) enrichment analysis (Fig. 2b, c), enabled the identification of biological processes associated with the main transcriptional patterns. This analysis identified four groups of genes associated with distinct biological processes. The first group (acclimation activated), including clusters 1 and 2, showed increased expression during acclimation. Genes in cluster 1 showed no response to cold induction, decreased expression during plant growth, and increased expression once acclimation was established. The main GO categories associated with this expression profile included flavonoid metabolic process, ribosome biogenesis and protein folding, together with glycogen metabolic process, response to starvation, cold acclimation and response to water deprivation (Fig. 2c; Table S1). Cluster 2 showed a similar pattern; however, gene expression also slightly increased in response to cold and freezing and reached full activation once acclimation was established. Some GO categories overlapped with those identified for cluster 1, such as response to water deprivation and cold acclimation, whereas others were specific to this cluster, including vitamin E metabolic process and regulation of circadian rhythm (Fig. 2b, c; Table S1). The second group includes clusters 4, 6 and 7, whose genes were downregulated once acclimation was established. Cluster 4 remained relatively stable following cold induction but showed higher expression in 18 day-old compared to 14 day-old seedlings and was repressed after acclimation. The GO categories associated with this cluster included photosynthesis, water transport, response to nitrate, response to nutrient levels and response to oxidative stress (Table S1). Genes in clusters 6 and 7 were active under control conditions and during cold induction, showing slight differences in the expression level between WT and *clf-29*. Both clusters showed decreased expression once acclimation was established. The GO categories associated with cluster 6 included light harvesting, indole glucosinolate metabolic process, and cell wall organization, whereas cluster 7 was enriched for cellular response to nutrient levels, root hair elongation, triterpenoid metabolic process and intracellular iron ion homeostasis (Fig. 2b, c; Table S1). Genes in cluster 3 were activated in response to cold and freezing, including after acclimation, and showed slightly higher expression levels in the *clf-29* mutant. The representative GO categories associated with this cluster included response to oxygen levels, to wounding, regulation of response to stress, ethylene-activated signaling pathway, seedling development, and protein autoubiquitination (Fig. 2b, c; Table S1). Clusters 5, 8, 9 and 10 displayed an expression profile characterized by consistent differences between WT and *clf-29* across conditions (*clf*-affected), with 5, 9 and 10 showing higher expression levels, while in cluster 8 a lower expression in the *clf-29* mutant background. These clusters contained a relatively small number of genes among the 4,000 most variable. Nevertheless, several GO categories associated with these clusters were

unique and particularly relevant to the focus of this study, including systemic acquired resistance (62), priming of response to stress, response to salicylic acid, regulation of response to stress, immune system process, and negative regulation of developmental processes (Fig. 2b, c; Table S1).

Differential expression and comparative analysis across the time course

To compare *clf-29* and WT gene expression profiles, differential expression was assessed between the two genotypes separately within each of the six experimental conditions, so that each comparison contrasts the two genotypes in plants of the same age and after the same temperature treatment (Fig. 3a-f). This design asks how far the mutant has drifted from the WT in a given state. Statistics and details of the up- and down-regulated genes identified in each condition are reported in Table S2. Functional enrichment of the genes differentially expressed between *clf-29* and the WT within each condition is shown in Fig. S1a-f (Table S3). Across the six conditions, 405 transcription factors belonging to 51 families are differentially expressed between *clf-29* and the WT (Fig. 3g, Fig. S1g-l, Table S4). Eight families are represented in all six conditions (63): MIKC_MADS, MYB, HD-ZIP, NAC, TCP, B3, GRAS and SRS. Four of these families are significantly enriched among the 405 relative to the 1,717 annotated *Arabidopsis* transcription factors (Fig. 3h): MIKC_MADS (22 out of 42), HB-other (6 out of 7), NF-YA (7 out of 10) and TALE (11 out of 21). Notably, no family associated with the canonical cold response is enriched.

The MADS-box signal is the strongest and the most consistent (64): nearly all 22 differentially expressed MIKC_MADS genes are up-regulated in the mutant, and four, *SHP2*, *SEP3*, *FLC* and *SEP4*, are derepressed in every condition (65–67), with the effect on *SHP2* reaching 8.65 log₂ units at AC_{Fr}, a roughly 400-fold increase (Fig. 3i). Two TCP genes involved in shoot branching, *BRC2* and *TCP1*, are derepressed in all conditions, and *BRC1* (68) in the four conditions other than the two acclimated states. All three belong to the CYC/TB1 class II TCP factors (*BRC1*/*TCP18*, *BRC2*/*TCP12* and *TCP1*), while the five differentially expressed CIN-clade members change weakly and mostly in the opposite direction. Among the 18 differentially expressed *homeodomain-leucine zipper* (HD-ZIP) genes, the direction of change follows the class, with classes I and II derepressed (12 of 13 up-regulated, strongest *ATHB54*) and all class III and IV members down-regulated, whereas the 18 NAC (*NAM*/*ATAF1,2*/*CUC2*) genes are predominantly up-regulated (16 of 18). Five of the six KNOX genes, canonical Polycomb targets (69–71), are up-regulated: *BP*/*KNAT1* and *KNAT3* in five conditions, and *STM*, *KNAT6* and *KNAT2* in the older or frozen samples.

Two families relevant to abiotic stress behave in opposite ways. Within the NAC family, 18 of 113 members are differentially expressed: *ANAC018* is derepressed in all six conditions and *NAC056* in five, both at moderate amplitude, whereas *ANAC019* is derepressed only at CI, NAc and NAc_{Fr} but with an effect three times larger (+ 2.06 to + 2.40). The WRKY family (72), by contrast, is essentially untouched: only 11 of 72 members are differentially expressed, none in more than two conditions, and six of them only at NAc. *ABO3*/*WRKY63*, an ABA-dependent regulator of drought and cold tolerance, is derepressed

only at CI (+ 2.83). This is not because *WRKY* genes are unresponsive: in the WT, *WRKY*s respond to every temperature shift, from 3 members at ACFr vs AC to 21 at AC vs NAc.

The genes downregulated in the mutant are consistently reduced across conditions (Fig. 3a-f). *CLF* itself is the strongest signal in every panel (-5.31 to -6.14 log₂ units, $\text{padj} < 10^{-68}$), which confirms the identity of the *clf-29* allele and provides an internal control for each comparison. Beyond it, *WOX1*, a regulator of leaf and floral organ polarity, is reduced in five of the six conditions (mean - 1.06); *SNZ*, a repressor of flowering, in four (mean - 1.31); and *ATMSRB6* in the conditions of the 18 day-old samples. Condition-specific reductions include the ABA-induced repressor *AITR1* at NC and AC, the bHLH transcription factor *CIB1* at CI and NAc, and the sugar transporter *AtSWEET4* (73) at ACFr. That *WOX1* and *SNZ* are among the most consistently reduced genes indicates that the developmental deregulation of the mutant is bidirectional.

Finally, the canonical cold-responsive genes are not derepressed in the mutant (Fig. 3i). *CBF3/DREB1A* is unchanged in all six conditions; *CBF1* is slightly reduced at CI and NAcFr (-0.75 and - 0.84) and *CBF2* at NAc (-1.13); *COR15A* and *RD29A* are reduced only at NAcFr (74, 75).

We performed an expression analysis, by RT-qPCR, to validate our results. Two genes were chosen as markers of the cold response: *CBF3*, as an early cold-responsive gene, and *COR15A*, as downstream target of the CBF factors whose transcript increases during acclimation. The results showed that, as expected, *CBF3* was strongly induced at CI and at NAcFr (524 and 417 fold in the WT, respectively), then the transcript level decreased in AC samples (50 fold), and a similar profile was shown in the corresponding *clf-29* mutant samples. *COR15A* showed a complementary profile, with the highest expression at AC and at ACFr (3186 and 2566 fold, respectively). These results are consistent with the RNA-seq data, confirming that the setup of the RNA-seq assay clearly separated the early cold response from established acclimation, and that the two genotypes share largely the same cold-induction profile, with only minor differences between the two assays at some time points (Fig. 3j, k). Two transcription factor-encoding genes were also validated: *ANAC019*, which belongs to the *clf*-specific sets, and *NAC056*, which is derepressed in the mutant irrespective of temperature. As for *ANAC019*, the expression profiles from both experiments are consistent, with a significant up-regulation in the non-acclimated conditions (CI, NAc and NAcFr), although the RT-qPCR revealed also significant differences in the NC and ACFr conditions. The transcript level of *NAC056* was higher in *clf-29* with respect to the WT across conditions, reaching 9.7-fold against 3.1-fold at CI and 9.7-fold against 1.5-fold at NAcFr, thus mirroring the results of the RNA-seq (Fig. 3j-m; Table S5). Taken together, the two genes describe the CLF-dependent programme as having a constitutive and a conditional part. A set of genes requires CLF for repression at all times and is derepressed further when the mark is reduced by cold, of which *NAC056* is the clearest example; a second, larger set is repressed adequately in control conditions and becomes CLF-dependent only when cold remodels the chromatin landscape, which is what the *clf*-specific sets capture and what *ANAC019* exemplifies.

***clf* -specific cold response genes are enriched in PRC2 targets**

To identify *clf*-specific genes of the cold response, we compared, within each genotype, its own response to each cold condition, rather than comparing the two genotypes inside a single condition. For each of the four cold treatments - early cold (CI versus NC), acclimation (AC versus NAc), freezing without acclimation (NAcFr versus NAc) and freezing after acclimation (ACFr versus AC) - differential expression was assessed, and the two gene lists were compared using Venn diagrams (Fig. 4a-d). The corresponding overlaps within the WT alone, shown separately for up- and down-regulated genes, are presented in Fig. S2. The sector responding only in *clf-29* defines the *clf*-specific set: 686 genes at CI, 2,571 at AC, 1,423 at NAcFr and 801 at ACFr (Fig. 4e). These sets contain 58, 147, 95 and 47 transcription factors respectively, dominated by the MYB, bHLH, C2H2 and ERF families, followed by WRKY and NAC - a composition that differs from the constitutively deregulated factors described above (Fig. 4f).

Functional enrichment separates the four cold conditions (Fig. 4g-j, Table S6). At CI the genes responding only in the mutant are enriched for hypoxia and decreased oxygen response (5.5 to 5.9-fold), while those down-regulated concern cell wall organization (5.0 to 6.8-fold). At AC the up-regulated set is enriched for RNA modification, chromatin organization and mRNA metabolism (2.0 to 2.6-fold). Freezing without acclimation gives vegetative-to-reproductive phase transition and reproductive shoot development among the up-regulated genes (2.4 to 3.2-fold), and lipid transport and circadian regulation among the down-regulated ones; after acclimation, freezing affects carbohydrate transport and secondary metabolism (3.4 to 9.9-fold). None of the *clf*-specific sets showed significant enrichment for canonical cold-acclimation GO terms.

To place these genes in a regulatory context, we inferred a co-expression network with GENIE3 (56) separately for each cold condition, using *clf*-specific transcription factors as candidate regulators and its *clf*-specific genes as targets (Fig. 4k-n). The panels show the ten regulators with the highest weight among all candidate regulators (58 at CI, 147 at AC, 95 at NAcFr and 47 at ACFr). At CI, these include the induced *ERF109*, *ERF71* and *ANAC019*. At acclimation, *MYB72* and the heat shock factor *HSFA7B* are reduced while the bHLH factor *CIB1* - strongly reduced in the mutant at CI - appears as an induced regulator. Freezing without acclimation is dominated by C2H2, NAC and WRKY factors, including *ANAC090* and *ZAT7* genes (76), and freezing after acclimation by ERF and C2H2 factors. Across all cold conditions the regulators are stress- and development-related rather than canonical CBF factor encoding genes.

We then asked whether these genes are candidate Polycomb targets, using the H3K27me3 profile of cold-treated WT seedlings generated by Faivre et al. (37) (Fig. 4o). Interestingly, the CI set is enriched: 18.9% of its 672 genes with annotated coordinates carry the mark against a genomic background of 11.0% (odds ratio 1.95, $p = 7.4 \times 10^{-10}$). On the contrary, AC is marginal (12.3% of 2,489 genes, odds ratio 1.16, $p = 2.9 \times 10^{-2}$) and neither freezing set differs from background (NAcFr 10.1%, ACFr 10.0%, both odds ratio 0.90, n.s.). The loci escaping control in *clf-29* are, therefore, preferentially Polycomb targets, consistent with lack of CLF releasing a subset of PRC2-repressed cold-responsive genes (Fig. 4). Restricting the four sets of Fig. 4a-d to genes that are both Polycomb targets (Fig. 4o) and significantly

misregulated in the mutant ($\text{padj} < 0.05$) results in 47 high-confidence cases (Table S7), including transcription factors de-repressed in *clf-29* (*ANAC019* and *SRS4* at CI; the MADS-box genes *SEP3* and *SHP1* and *TCP1* at AC) and the sugar transporter encoding gene *AtSWEET10*, de-repressed in all six conditions ($\log_2 \text{FC} + 2.5$ to $+ 6.8$; $+4.8$ at NAcFr, $\text{padj} 7 \times 10^{-34}$) and the stress factor encoding gene *ZAT7*, while *AtSWEET11* is reduced (77) ($\log_2 \text{FC} -0.79$). The acclimation set was uniformly up-regulated in the mutant (15/15 genes), consistent with derepression due to lack of CLF.

Finally, we asked whether these genes are enriched for conserved *cis*-regulatory elements, including the telobox motifs proposed to recruit CLF/SWN-PRC2 (33). Scanning their promoters against the JASPAR (78) collection (702 matrices at CI, 703 at AC) returned no telobox signature at any cold condition (Fig. S3, Table S8): at CI, the TRB motifs are, if anything, under-represented (odds ratio 0.77) and at AC marginally enriched but not significant. The early cold promoters are not featureless: 68 of the 702 matrices tested are enriched, and they belong to coherent families rather than being scattered. The *AT-hook* (AHL (79)) and *TCX/CPP* (80) matrices are enriched in full, homeobox families (81) in more than half, while large families such as *MYB* and *NAC* contribute almost nothing (odds ratios 1.26 to 1.62; Fig. S4, Tables S9 and S10). Thus, although the *clf*-specific genes are enriched in PRC2 targets, their promoters do not carry a detectable telobox/TRB signature. This indicates that PRC2 recruitment to these cold-responsive loci does not rely on canonical teloboxes, but may instead proceed through other *cis*-elements, through TRB binding that is not captured by the consensus motif, or through recruitment mechanisms independent of sequence motifs.

A cold-induced rescue effect: cold attenuates *clf*-dependent derepression

The largest difference between the two genotypes is observed at NAc, where 3,068 genes are up-regulated in *clf-29* with respect to the WT ($\text{padj} < 0.05$), with a mean *clf-29* versus WT difference of $+ 0.76$ $\log_2$ units. This derepression reflects the missing CLF activity: CLF regulates a developmental programme that advances quickly at this stage of seedling growth (66, 67). On these 3,068 genes, the mean difference between *clf-29* and the WT is already $+ 0.24$ at NC and rises to $+ 0.76$ at NAc. We noticed that cold markedly reduced this difference between the two genotypes, and asked how this happens: to what extent, whether a short exposure is enough, and whether the two treatments act on the same genes.

After four days at 4°C the two genotypes are nearly indistinguishable (Fig. 5a). Indeed, their expression profiles converge even more than in the 14 day-old NC plants ($+ 0.18$ at AC against $+ 0.24$ at NC): acclimation does not simply hold development at the 14 day-old stage, it seems to actively bring the mutant back towards the WT. A short cold induction has a similar effect, but interestingly only in 18 day-old plants: three hours at 4°C do not reduce the difference in 14 day-old plants ($+ 0.30$ at CI against $+ 0.24$ at NC), whereas the temperature decrease applied at 18 days halves it (from $+ 0.76$ at NAc to $+ 0.38$ at NAcFr).

The two cold treatments at 18 days, short (NAcFr) and prolonged (AC), act largely on the same genes: for 1,522 of them the difference decreases by more than 0.3 $\log_2$ units under both (Fig. 5b). Acclimation

has a broader effect, affecting a further 877 genes by itself, whereas the short treatment only 173. The short cold induction at 14 days acts instead on different genes: those affected between NC and CI share only 15 genes with those affected between NAc and NAcFr, and the two sets differ in chromatin state: the CI set is enriched for H3K27me3 in the WT map (37) (Fig. 4o; 29.6% against a genomic background of 11.0%) whereas the NAcFr set is depleted (7.2%; Fig. S5d).

Since the four days at 4°C also slow growth, age and cold treatment are not fully independent in this design, and the derepressed genes are likely to respond both to development and to cold. Whatever the relative contribution of the two, the outcome is the same: after four days at 4°C the transcriptome of *clf-29* is almost indistinguishable from that of the WT.

A genotypexcondition interaction test localises CLF-dependent remodelling to acclimation

To evaluate formally whether the effect of CLF on the cold response varies with developmental stages and/or cold conditions, rather than depending on CLF activity itself, we fitted a genotypexcondition interaction model in DESeq2 (82) and tested the interaction at each cold transition (Wald test). Across the four cold conditions the test was significant almost exclusively at acclimation: 1,358 genes for the AC-vs-NAc comparison, versus 1 (CI-vs-NC), 0 (ACFr-vs-AC) and 7 (NAcFr-vs-NAc) (Fig. 6a-e). Only the acclimated condition therefore yields a gene set large enough for downstream analysis. The AC interaction set was enriched for ribosome biogenesis, rRNA processing and chromatin organization among up-regulated genes and for mitochondrial respiratory-chain and ATP-synthesis functions among down-regulated genes (Fig. 6d, Table S11). No JASPAR matrix, including the three telobox/TRB motifs, was enriched in the promoters of the interaction genes (Fig. 6c).

Interestingly, these acclimation interaction genes were not direct PRC2 targets. They were significantly depleted, not enriched, for H3K27me3 in the WT background according to (37) (5.5% versus a genome background of 11.0%, odds ratio 0.46, $p = 1.3 \times 10^{-12}$), and their promoters showed no significant enrichment for telobox/TRB recruitment motifs (TRB1 odds ratio 1.19, not significant after multiple-testing correction). These genes therefore represent a class of metabolic and chromatin-regulatory genes that are not themselves H3K27me3-marked, distinct from the marked Polycomb targets that characterise the *clf*-specific sets. To test whether lack of CLF affects freezing tolerance, we performed a phenotypic analysis of the *clf-29* mutant under cold and freezing stress. We used a set-up adapted from Perea-Resa et al. (42), briefly: 14 day-old plants were grown in soil, then assigned to one of three regimes: no cold (NC) as an untreated control, at 22°C; acclimation (AC), 4°C for four days; or no acclimation (NA), kept at 22°C for four days. Acclimated and non-acclimated plants were then frozen at -7°C for six hours, so that NA reports freezing injury in the absence of acclimation. Plants were returned to standard conditions for 7 days of recovery, after which survival was scored (Fig. 6f). Interestingly, survival was higher in *clf-29* than in the WT, under all conditions, and the analysis of variance detected a significant, although slight, genotype effect ($F = 4.84$, $p = 0.035$) alongside the expected strong effect of the temperature regime ($F = 9.07$, $p = 0.0008$). There was no genotype $\times$ regime interaction ($p = 0.75$),

indicating that the magnitude of the mutant's advantage did not differ detectably between conditions. Nevertheless, the only significant difference was in the acclimated condition: 84.8% of *clf-29* plants survived against 55.4% of the WT, while survival was 50.0% versus 33.3% after freezing without acclimation (Fig. 6g, h). In the absence of an interaction the advantage cannot be formally assigned to acclimation, and the marked difference in size and developmental stage between the genotypes (Fig. 6h) may contribute to it.

Polycomb machinery and the CBF regulon in *clf*

We next asked whether the Polycomb machinery itself and the canonical CBF module behave differently in the *clf-29* mutant background. Comparing the cold response of chromatin regulators (83) between genotypes (Difference-Of-Differences, DoD), six factors were significantly more cold-responsive in *clf-29* than in the WT in the acclimated condition: the H3K27me3 demethylases *REF6* (84) (DoD + 0.72, $p = 0.041$) and *ELF6* (85) (+ 0.56, $p = 0.022$), the PRC2 accessory factor *VIL1* (39) (+ 0.47, $p = 0.013$), the H3K4 demethylase *JMJ14* (86, 87) (+ 0.36, $p = 0.008$) and the core subunits *EMF2* (88) (+ 0.32, $p = 0.031$) and *VRN2* (89) (+ 0.23, $p = 0.046$) (Fig. 7a, b). *SWN* (29) (+ 0.41) and *LHP1* (90) (+ 0.44) show the same positive, although not significant, trend, and *CLF* itself is reduced (-0.71 , $p = 0.036$), as expected for the mutated locus. The effect is strongest at acclimation: at early induction no factor differs between genotypes, and after freezing only *VIL1* and *JMJ14* remain significant (Fig. 7a, b). This suggests a compensatory up-regulation of Polycomb-antagonising and accessory activities in the absence of CLF. In contrast, the CBF-COR module was largely unchanged: the CBF-COR induction pathway overlapped between genotypes, with only a mild reduction of *CBF1* at cold induction ($\log_2$ FC -0.75, $\text{padj } 0.003$) and no CLF-dependent change in *CBF2* ($\text{padj } 0.58$) or *CBF3* ($\text{padj } 0.91$), and no derepression of the CBF cluster at acclimation (Fig. 7c-f). The CLF-dependent programme seems therefore largely distinct from the canonical CBF cold regulon.

Discussion

The CBF module is the canonical and the best characterized entry point of cold stress responses in plants: three tandemly arranged genes, *CBF1* to *CBF3*, are induced within hours of cold exposure and in turn activate the *COR* regulon, displaying a transient induction, declining once acclimation is established (7, 12, 15). Polycomb Repressive Complex 2 has recently been shown to act on this module in at least two ways. Gómez-Martínez et al. showed that the decline of *CBF3* is epigenetically enforced: the cold-inducible long noncoding RNA *SVALKa*, transcribed antisense in the *CBF3-CBF1* intergenic region, recruits PRC2 to deposit H3K27me3 over the *CBF3* coding region and, in a *clf-28 swn-7* double mutant background, *CBF3* induction is no longer restrained (41). Independently, Kim et al. showed that the Polycomb protein *VIL1* deposits H3K27me3 across the three clustered *CBF* genes maintaining a repressive chromatin conformation, with lack of *VIL1* increasing chromatin accessibility and raising *CBF1* expression at low ambient temperature (40). Our transcriptomic data place CLF upstream of a condition-restricted gene programme that is remodelled across cold acclimation, while leaving the canonical *CBF-COR* cold regulon CLF-independent. Indeed, expression of *CBF2* and *CBF3* is essentially

unaffected in the *clf-29* mutant compared to the WT, while *CBF1* shows a mild reduction at cold induction and after freezing without acclimation. The downstream *COR* genes behave in a similar way: *COR47*, *KIN1* and *KIN2* are up-regulated only in the 18 day-old control (NAC), among the 3,068 genes up-regulated in that condition. At first sight this looks like a contradiction between the two datasets, since the locus placed under Polycomb control is precisely the one we find unaffected in the mutant, yet it is readily explained: Gómez-Martínez et al. observed *CBF3* derepression in the *clf-28 swn-7* double mutant, in which both PRC2 catalytic subunits are disabled (41), unlike our single *clf-29* background, where SWN remains functional; while Kim et al. (40) showed that VIL1 provides a further CLF-independent route to the same cluster. The single mutant is therefore informative as it separates the loci that depend on CLF alone from those, like the *CBF cluster*, where several Polycomb activities converge. A caveat concerns timing: in the *clf-28 swn-7* double mutant the excess of *CBF1* and *CBF3* transcripts is most evident after 24 h at 4°C (41), a window that lies between our 3 h and 4 d samples, thus a transient contribution of CLF at that stage cannot be excluded.

Besides the CBF module, two findings characterise the genes that CLF does control. First, the genes that respond to cold specifically in the mutant (*clf*-specific sets) carry H3K27me3 in the WT more than genes genome-wide – 18.9% against 11.0% at CI (odds ratio 1.95) – identifying them as candidate PRC2 targets. Second, their promoters are not enriched for telobox/TRB motifs: scanning the *clf*-specific promoters against a genomic background gave no significant enrichment at any point. Nevertheless, 68 of the 702 motifs tested are enriched in the same promoters, and they fall into coherent families dominated by AHL and TCX/CPP. AHL proteins bind AT-rich matrix-attachment regions, and TCX/CPP factors carry CXC domains; both are associated with chromatin architecture rather than with cold signalling. PRC2 recruitment to these loci therefore does not appear to rely on canonical teloboxes, and the *cis*-signature points to an architectural rather than a stress-signalling route.

Modelling the genotypexcondition interaction localised CLF-dependent remodelling almost exclusively to acclimation. These interaction genes are enriched for ribosome biogenesis and mitochondrial energy metabolism and are, unlike the *clf*-specific sets, depleted rather than enriched for H3K27me3, so they are unlikely to be direct PRC2 targets. The genes that remain misregulated after acclimation tell the complementary story: among those up-regulated in *clf-29*, the fraction marked by H3K27me3 rises from 13% at NAC, close to the genomic background, to 48% at AC (Table S2). What the interaction genes share is a growth signature: ribosome-related categories are also lower in *clf-29* at control temperature and no longer differ after acclimation. Since cold itself reprogrammes ribosome biogenesis (91, 92), the interaction most simply reflects a difference in growth state between the genotypes at 22°C that cold removes.

Two points bear on the size of the CLF-dependent effect, and they act in opposite directions: time spent growing at control temperature and exposure to cold. Four extra days at 22°C raise the number of differentially expressed genes more than 6-fold, from 983 in 14 day-old plants to 6,358 in 18 day-old plants, whereas the same four days at 4°C leave 164. Lack of CLF therefore does not create a fixed deficit that time reveals; it creates a deregulation that builds up while the plant grows in control

temperature and that cold holds back. This build-up is expected: CLF represses *AG*, *SEP3*, *FLC* and *FT* during vegetative growth (65–67), and at 18 days the senescence genes *ORE1* and *NAP* are already up-regulated in *clf-29*, so chronological and developmental age are not equivalent in our design. Developmental arrest alone, however, cannot explain the cold effect: most constitutively derepressed genes are less derepressed after acclimation than at 14 days. Whether cold prevents the deregulation from arising or reverses it once present cannot be settled here. Nevertheless, one observation points to the second possibility: freezing applied to non-acclimated plants reduces a difference that is already established (+ 0.76 to + 0.38), so cold can act on deregulation that has already occurred. Because this attenuation takes place in plants that lack CLF, it cannot be mediated by CLF itself. Two explanations remain open: at low temperature another PRC2 activity, most plausibly SWN, may restore repression at part of these loci, or the reduced growth and transcription at 4°C may reduce the secondary effects of the mutation without any change in chromatin. Our data do not distinguish between them. The chromatin dataset we used (37) shows that short-term cold redistributes H3K4me3 and H3K27me3 on largely distinct sets of genes, that these changes correlate only weakly with expression, and that in the *clf* mutant many cold-regulated genes lose H3K27me3 while their activation is essentially unchanged. A second study found that cold induces the joint deposition of H3K4me3 and H3K27me3 over about two thousand actively expressed genes, whose expression is largely independent of the level of the two marks, and that the deposition of H3K27me3 fails in *clf-28* and *lhp1* mutants, implicating CLF in this cold-induced gain (93). H3K27me3 therefore looks modulatory rather than switch-like, in agreement with the earlier observation that the activation of *COR15A* and *ATGOLS3* is not inhibited by H3K27me3 but rather leads to its removal (36), and with dehydration-memory genes, which are transcribed despite carrying the mark (38). The phenotype points the same way. In our freezing assays the *clf-29* mutant survived more than the WT, with a significant genotype effect in the analysis of variance ($F = 4.84$, $p = 0.035$) and no genotype $\times$ regime interaction; the only individually significant difference was in acclimated plants, where 84.8% of *clf-29* survived against 55.4% of the WT. Transcriptome and phenotype thus point to the same window: acclimation is when CLF constrains the response, and the improved survival of the mutant is consistent with the release of that constraint.

Taken together, these observations are consistent with a division of role among PRC2 subunits rather than with a single repressive switch. CLF holds back a broad, developmental programme that builds up as the plant grows in normal conditions and is restrained by cold, and the single mutant reveals it because no other subunit covers it; the CBF module lies outside that programme, protected by SWN and by the parallel routes through VIL1 and SVALK1, which would explain why removing CLF leaves the canonical cold regulon intact yet still improves survival after acclimation. This matters because it separates the cold response into a part that Polycomb guards redundantly and a part that depends on CLF alone, and it identifies acclimation as the stage at which chromatin repression limits how far the response can go.

Conclusions

We set out to establish what the Polycomb methyltransferase CLF contributes to the cold response of *Arabidopsis*. CLF is not needed to switch on the canonical *CBF* pathway, in line with the redundant Polycomb control of the *CBF* locus. What plants lacking CLF show instead is a broad misregulation of gene expression that builds up as they grow at normal temperature, and that cold acclimation almost completely removes. Only during acclimation does part of the cold response depend on CLF, and it involves growth-related genes that are depleted of H3K27me3: this effect is therefore largely indirect. The mutant also survives freezing better than the WT. Together, these findings show that lack of CLF matters least for the transcriptome during acclimation. Acclimation is therefore a good condition in which to separate the direct targets of CLF from the indirect effects of its loss, parting the H3K27me3-dependent targets of CLF apart from the secondary consequences of altered development. Our inferences on H3K27me3 rest, however, on chromatin maps of the WT.

Abbreviations

General

WT	wild type
Col-0	Columbia-0, the wild-type accession
DEG	differentially expressed gene
DEA	differential expression analysis
GO	Gene Ontology
PCA	principal component analysis
VST	variance-stabilising transformation
RT-qPCR	reverse-transcription quantitative PCR
ChIP-seq	chromatin immunoprecipitation followed by sequencing
TSS	transcription start site
DoD	difference of differences

Experimental conditions

NC	no cold, 14 day-old plants
CI	cold induction, 3 h at 4 °C
NAC	non-acclimated 18 day-old control
AC	acclimated, four days at 4 °C
NACFr	freezing without prior acclimation
ACFr	freezing after acclimation

Chromatin and Polycomb

PRC2	Polycomb Repressive Complex 2
<i>CLF</i>	CURLY LEAF
<i>SWN</i>	SWINGER
<i>MEA</i>	MEDEA
<i>EMF2</i>	EMBRYONIC FLOWER 2
<i>VRN2</i>	VERNALIZATION 2
<i>VIL1</i>	VERNALIZATION INSENSITIVE 3-LIKE 1
<i>LHP1</i>	LIKE HETEROCHROMATIN PROTEIN 1
<i>REF6</i>	RELATIVE OF EARLY FLOWERING 6
<i>ELF6</i>	EARLY FLOWERING 6
<i>JMJ14</i>	JUMONJI DOMAIN-CONTAINING PROTEIN 14
H3K27me3	tri-methylation of histone H3 at lysine 27
H3K4me3	tri-methylation of histone H3 at lysine 4
H3K36me3	tri-methylation of histone H3 at lysine 36
TRB	TELOMERE REPEAT-BINDING factor
TCX/CPP	tesmin/TSO1-like CXC domain family, also called CPP
CXC	cysteine-rich domain shared by TCX/CPP proteins

Cold-response pathway

<i>CBF1-CBF3</i>	C-REPEAT BINDING FACTOR 1 to 3, also DREB1B, DREB1C and DREB1A
<i>COR15A</i>	COLD-REGULATED 15A
<i>RD29A</i>	RESPONSIVE TO DESICCATION 29A
<i>ICE1</i>	INDUCER OF CBF EXPRESSION 1
<i>PIF</i>	PHYTOCHROME-INTERACTING FACTOR
<i>SVALKA</i>	the CBF3 antisense long non-coding RNA
CRT/DRE	C-repeat / dehydration-responsive element
ABA	abscisic acid

Transcription-factor families

MIKC_MADS	MADS-box family, MIKC type
bHLH	basic helix-loop-helix
ERF	ETHYLENE RESPONSE FACTOR
NAC	NAM, ATAF and CUC family
WRKY	WRKY-domain family
C2H2	C2H2 zinc-finger family
TCP	TEOSINTE BRANCHED1, CYCLOIDEA, PCF family
HD-ZIP	homeodomain-leucine zipper
TALE/KNOX	three-amino-acid-loop-extension / KNOTTED-like homeobox
SRS	SHI-RELATED SEQUENCE
GRAS	GAI, RGA and SCR family
NF-YA	NUCLEAR FACTOR Y, subunit A
AHL	AT-HOOK MOTIF NUCLEAR LOCALIZED

Genes cited in the text

<i>ANAC019, ANAC090</i>	NAC-domain transcription factors 019 and 090
<i>NAC056</i>	NAC-domain transcription factor 056
<i>SHP1</i>	SHATTERPROOF 1 (AGL1)
<i>SEP3, SEP4</i>	SEPALLATA 3 (AGL9) and 4
<i>SHP2</i>	SHATTERPROOF 2
<i>STM, BP, KNAT1-KNAT6</i>	SHOOT MERISTEMLESS, BREVIPEDICELLUS and KNOTTED-like genes
<i>WOX1</i>	WUSCHEL-RELATED HOMEODOMAIN 1
<i>CIB1</i>	CRYPTOCHROME-INTERACTING bHLH 1
<i>SNZ</i>	SCHNARCHZAPFEN
<i>ZAT7</i>	ZINC FINGER OF ARABIDOPSIS THALIANA 7
<i>AtSWEET4, 10, 11</i>	SUGARS WILL EVENTUALLY BE EXPORTED TRANSPORTER family
<i>FLC</i>	FLOWERING LOCUS C
<i>ACT2</i>	ACTIN 2, the RT-qPCR reference gene
<i>ATMYB72</i>	MYB-domain transcription factor 72
<i>HSFA7B</i>	HEAT SHOCK FACTOR A7B
<i>BRC1, BRC2</i>	BRANCHED 1 and 2
<i>SRS4</i>	SHI-RELATED SEQUENCE 4
<i>ATHB54</i>	ARABIDOPSIS THALIANA HOMEODOMAIN 54
<i>ATMSRB6</i>	METHIONINE SULFOXIDE REDUCTASE B6
<i>AITR1</i>	ABA-INDUCED TRANSCRIPTION REPRESSOR 1
<i>AtSWEET10, AtSWEET11</i>	SUGARS WILL EVENTUALLY BE EXPORTED TRANSPORTER 10 and 11
<i>KNAT2, KNAT3</i>	KNOTTED1-LIKE HOMEODOMAIN 2 and 3

Software and databases

DESeq2	R package for differential expression analysis
GENIE3	gene network inference with ensembles of trees
PlantTFDB	Plant Transcription Factor Database
JASPAR	database of transcription-factor binding profiles
MEME/FIMO	motif discovery suite and its motif-scanning tool
TAIR10	The Arabidopsis Information Resource, genome release 10

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Author Contribution

All authors reviewed the manuscript. Conceptualization, CL and PV; Writing—original draft preparation, CL and PV; Data analysis, CL, AL, GFF, GP, GGT, and AC; Interpretation and discussion of the results, CL, AL, FDO, MP, RDI, AC, and PV; Critical review and editing, AL, RDI, FDO, MP, GFF, and AC; Supervision, PV. All authors read and approved this version of the manuscript.

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Data Availability

RNA-Seq data were deposited into the Gene Expression Omnibus database under accession number GSE348022 and are available at the following URL: <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE348022>

References

1. Zhu JK. Abiotic stress signaling and responses in plants. *Cell*. 2016;167:313–24.
2. Thomashow MF. Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. *Annu Rev Plant Physiol Plant Mol Biol*. 1999;50:571–99.
3. Shi Y, Ding Y, Yang S. Molecular regulation of CBF signaling in cold acclimation. *Trends Plant Sci*. 2018;23:623–37.
4. Ding Y, Shi Y, Yang S. Molecular regulation of plant responses to environmental temperatures. *Mol Plant*. 2020;13:544–64.
5. Chinnusamy V, Ohta M, Kanrar S, Lee BH, Hong X, Agarwal M, et al. ICE1: a regulator of cold-induced transcriptome and freezing tolerance in Arabidopsis. *Genes Dev*. 2003;17:1043–54.
6. Kim YS, Lee M, Lee JH, Lee HJ, Park CM. The unified ICE-CBF pathway provides a transcriptional feedback control of freezing tolerance during cold acclimation in Arabidopsis. *Plant Mol Biol*. 2015;89(1–2):187–201.
7. Gilmour SJ, Zarka DG, Stockinger EJ, Salazar MP, Houghton JM, Thomashow MF. Low temperature regulation of the Arabidopsis CBF family of AP2 transcriptional activators as an early step in cold-induced COR gene expression. *Plant J*. 1998;16:433–42.
8. Park S, Lee CM, Doherty CJ, Gilmour SJ, Kim Y, Thomashow MF. Regulation of the Arabidopsis CBF regulon by a complex low-temperature regulatory network. *Plant J*. 2015;82:193–207.
9. Jia Y, Ding Y, Shi Y, Zhang X, Gong Z, Yang S. The cbfs triple mutants reveal the essential functions of CBFs in cold acclimation and allow the definition of CBF regulons in Arabidopsis. *New Phytol*. 2016;212:345–53.
10. Jaglo-Ottosen KR, Gilmour SJ, Zarka DG, Schabenberger O, Thomashow MF. Arabidopsis CBF1 overexpression induces COR genes and enhances freezing tolerance. *Sci (80-)*. 1998;280:104–6.
11. Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, et al. Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression in Arabidopsis. *Plant Cell*. 1998;10:1391–406.

12. Gilmour SJ, Sebolt AM, Salazar MP, Everard JD, Thomashow MF. Overexpression of the Arabidopsis CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. *Plant Physiol.* 2000;124:1854–65.
13. Novillo F, Alonso JM, Ecker JR, Salinas J. CBF2/DREB1C is a negative regulator of CBF1/DREB1B and CBF3/DREB1A expression and plays a central role in stress tolerance in Arabidopsis. *Proc Natl Acad Sci USA.* 2004;101:3985–90.
14. Novillo F, Medina J, Salinas J. Arabidopsis CBF1 and CBF3 have a different function than CBF2 in cold acclimation and define different gene classes in the CBF regulon. *Proc Natl Acad Sci USA.* 2007;104:21002–7.
15. Barrero-Gil J, Salinas J. Gene regulatory networks mediating cold acclimation: the CBF pathway. *Adv Exp Med Biol.* 2018;1081:3–22.
16. Dong MA, Farré EM, Thomashow MF. CIRCADIAN CLOCK-ASSOCIATED 1 and LATE ELONGATED HYPOCOTYL regulate expression of the C-REPEAT BINDING FACTOR (CBF) pathway in Arabidopsis. *Proc Natl Acad Sci USA.* 2011;108:7241–6.
17. Lee CM, Thomashow MF. Photoperiodic regulation of the C-repeat binding factor (CBF) cold acclimation pathway and freezing tolerance in Arabidopsis thaliana. *Proc Natl Acad Sci USA.* 2012;109:15054–9.
18. Jiang B, Shi Y, Peng Y, Jia Y, Yan Y, Dong X, et al. Cold-induced CBF-PIF3 interaction enhances freezing tolerance by stabilizing the phyB thermosensor in Arabidopsis. *Mol Plant.* 2020;13:894–906.
19. Kindgren P, Ard R, Ivanov M, Marquardt S. Transcriptional read-through of the long non-coding RNA SVALKA governs plant cold acclimation. *Nat Commun.* 2018;9:4561.
20. Sun J, Zhao X, Fu D, Shi Y, Yang S, Qi Y, et al. An antisense RNA forms R-loop to facilitate the transcription of CBF genes and plant cold acclimation. *Dev Cell.* 2025;60:2445–e24545.
21. Kumar M, Rani K. Epigenomics in stress tolerance of plants under the climate change. *Mol Biol Rep.* 2023;50:6201–16.
22. Scintu D, Scacchi E, Cazzaniga F, Vinciarelli F, De Vivo M, Shtin M, et al. microRNA165 and 166 modulate response of the Arabidopsis root apical meristem to salt stress. *Commun Biol.* 2023;6:834.
23. Tie J, Xu T, Cheng L, Li T. Histone acetyltransferases as integrative epigenetic regulators of plant abiotic stress responses. *Plant Cell Rep.* 2026;45:227.
24. Shah SA, Chowhan A, Aslam S. Non-coding RNAs as central regulators of phytohormone signaling in plant abiotic stress responses. *Plant Mol Biol.* 2026;116:76.
25. Zhang X, Clarenz O, Cokus S, Bernatavichute YV, Pellegrini M, Goodrich J, et al. Whole-genome analysis of histone H3 lysine 27 trimethylation in Arabidopsis. *PLoS Biol.* 2007;5:e129.
26. Lafos M, Kroll P, Hohenstatt ML, Thorpe FL, Clarenz O, Schubert D. Dynamic regulation of H3K27 trimethylation during Arabidopsis differentiation. *PLoS Genet.* 2011;7:e1002040.

27. Godwin J, Farrona S. The importance of networking: plant Polycomb repressive complex 2 and its interactors. *Epigenomes*. 2022;6:8.
28. Xu F, Zhang D, Le L, Pu L. Polycomb and trithorax: Their yin-yang dynamics in plants. *Mol Plant*. 2024;17(6):845–7.
29. Chanvivattana Y, Bishopp A, Schubert D, Stock C, Moon YH, Sung ZR, et al. Interaction of Polycomb-group proteins controlling flowering in Arabidopsis. *Development*. 2004;131:5263–76.
30. Schubert D, Primavesi L, Bishopp A, Roberts G, Doonan J, Jenuwein T, et al. Silencing by plant Polycomb-group genes requires dispersed trimethylation of histone H3 at lysine 27. *EMBO J*. 2006;25:4638–49.
31. Shu J, Chen C, Li C, Cui Y. The complexity of PRC2 catalysts CLF and SWN in plants. *Biochem Soc Trans*. 2020;48:2779–89.
32. Xiao J, Jin R, Yu X, Shen M, Wagner JD, Pai A, et al. Cis and trans determinants of epigenetic silencing by Polycomb repressive complex 2 in Arabidopsis. *Nat Genet*. 2017;49:1546–52.
33. Zhou Y, Wang Y, Krause K, Yang T, Dongus JA, Zhang Y, et al. Telobox motifs recruit CLF/SWN-PRC2 for H3K27me3 deposition via TRB factors in Arabidopsis. *Nat Genet*. 2018;50:638–44.
34. Yang H, Howard M, Dean C. Antagonistic roles for H3K36me3 and H3K27me3 in the cold-induced epigenetic switch at Arabidopsis FLC. *Curr Biol*. 2014;24:1793–7.
35. Yang H, Berry S, Olsson TSG, Hartley M, Howard M, Dean C. Distinct phases of Polycomb silencing to hold epigenetic memory of cold in Arabidopsis. *Sci (80-)*. 2017;357:1142–5.
36. Kwon CS, Lee D, Choi G, Chung WI. Histone occupancy-dependent and -independent removal of H3K27 trimethylation at cold-responsive genes in Arabidopsis. *Plant J*. 2009;60:112–21.
37. Faivre L, Kinscher NF, Kuhlmann AB, Xu X, Kaufmann K, Schubert D. Cold stress induces rapid gene-specific changes in the levels of H3K4me3 and H3K27me3 in Arabidopsis thaliana. *Front Plant Sci*. 2024;15:1390144.
38. Liu N, Fromm M, Avramova Z. H3K27me3 and H3K4me3 chromatin environment at super-induced dehydration stress memory genes of Arabidopsis thaliana. *Mol Plant*. 2014;7:502–13.
39. Sung S, Schmitz RJ, Amasino RM. A PHD finger protein involved in both the vernalization and photoperiod pathways in Arabidopsis. *Genes Dev*. 2006;20:3244–8.
40. Kim J, Jeong RE, Sung S. Chromatin remodeling at the C-REPEAT BINDING FACTOR cluster controls growth retardation at low ambient temperature. *Plant Cell*. 2026;38:koag007.
41. Gómez-Martínez D, Barrero-Gil J, Tranque E, Ruiz MF, Catalá R, Salinas J. SVALKA-POLYCOMB REPRESSIVE COMPLEX2 module controls C-REPEAT BINDING FACTOR3 induction during cold acclimation. *Plant Physiol*. 2024;195:1152–60.
42. Perea-Resa C, Catalá R, Salinas J. Identification of Arabidopsis mutants with altered freezing tolerance. *Methods Mol Biol*. 2020;2156:85–97.
43. Seabold S, Perktold J. statsmodels: econometric and statistical modeling with Python. In: *Proceedings of the 9th Python in Science Conference*. 2010. pp. 92–6.

44. Virtanen P. others. SciPy 1.0: fundamental algorithms for scientific computing in Python. *Nat Methods*. 2020;17:261–72.
45. Illumina. FASTQ Toolkit [Internet]. 2026. Available from: <https://www.illumina.com/products/by-type/informatics-products/basespace-sequence-hub/apps/fastq-toolkit.html>
46. Patro R, Duggal G, Love MI, Irizarry RA, Kingsford C. Salmon provides fast and bias-aware quantification of transcript expression. *Nat Methods*. 2017;14:417–9.
47. Yates AD, Allen J, Amode RM, Azov AG, Barba M, Becerra A, et al. Ensembl Genomes 2022: an expanding genome resource for non-vertebrates. *Nucleic Acids Res*. 2022;50:D996–1003.
48. Soneson C, Love MI, Robinson MD. Differential analyses for RNA-seq: transcript-level estimates improve gene-level inferences. *F1000Res*. 2015;4:1521.
49. Carlson M. org.At.tair.db: genome wide annotation for Arabidopsis. R package version 3.22.0. 2023.
50. Pedregosa F, Varoquaux G, Gramfort A, Michel V, Thirion B, Grisel O, et al. Scikit-learn: machine learning in Python. *J Mach Learn Res*. 2011;12:2825–30.
51. Hunter JD. Matplotlib: a 2D graphics environment. *Comput Sci Eng*. 2007;9:90–5.
52. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc Ser B*. 1995;57:289–300.
53. Jin J, Tian F, Yang DC, Meng YQ, Kong L, Luo J, et al. PlantTFDB 4.0: toward a central hub for transcription factors and regulatory interactions in plants. *Nucleic Acids Res*. 2017;45:D1040–5.
54. Wu T, Hu E, Xu S, Chen M, Guo P, Dai Z, et al. clusterProfiler 4.0: a universal enrichment tool for interpreting omics data. *Innov*. 2021;2:100141.
55. Yu G, Li F, Qin Y, Bo X, Wu Y, Wang S. GOSemSim: an R package for measuring semantic similarity among GO terms and gene products. *Bioinformatics*. 2010;26:976–8.
56. Huynh-Thu VA, Irtuthum A, Wehenkel L, Geurts P. Inferring regulatory networks from expression data using tree-based methods. *PLoS ONE*. 2010;5:e12776.
57. Harris CR, editor. others. Array programming with NumPy. *Nature*. 2020;585:357–62.
58. Grant CE, Bailey TL, Noble WS. FIMO: scanning for occurrences of a given motif. *Bioinformatics*. 2011;27:1017–8.
59. Bailey TL, Johnson J, Grant CE, Noble WS. The MEME Suite. *Nucleic Acids Res*. 2015;43:W39–49.
60. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods*. 2001;25:402–8.
61. Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible WR. Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. *Plant Physiol*. 2005;139:5–17.
62. Singkaravanit-Ogawa S, Kosaka A, Kitakura S, Uchida K, Nishiuchi T, Ono E, et al. Arabidopsis CURLY LEAF functions in leaf immunity against fungal pathogens by concomitantly repressing SEPALLATA3 and activating ORA59. *Plant J*. 2021;108:1005–19.

63. Qu LJ, Zhu YX. Transcription factor families in Arabidopsis: major progress and outstanding issues for future research. *Curr Opin Plant Biol.* 2006;9(5):544–9.
64. Smaczniak C, Immink RGH, Angenent GC, Kaufmann K. Developmental and evolutionary diversity of plant MADS-domain factors: insights from recent studies. *Development.* 2012;139:3081–98.
65. Goodrich J, Puangsomlee P, Martin M, Long D, Meyerowitz EM, Coupland G. A Polycomb-group gene regulates homeotic gene expression in Arabidopsis. *Nature.* 1997;386:44–51.
66. Jiang D, Wang Y, Wang Y, He Y. Repression of FLOWERING LOCUS C and FLOWERING LOCUS T by the Arabidopsis Polycomb repressive complex 2 components. *PLoS ONE.* 2008;3:e3404.
67. Lopez-Vernaza M, Yang S, Müller R, Thorpe F, de Leau E, Goodrich J. Antagonistic roles of SEPALLATA3, FT and FLC genes as targets of the Polycomb group gene CURLY LEAF. *PLoS ONE.* 2012;7:e30715.
68. Aguilar-Martínez JA, Poza-Carrión C, Cubas P. Arabidopsis BRANCHED1 acts as an integrator of branching signals within axillary buds. *Plant Cell.* 2007;19:458–72.
69. Katz A, Oliva M, Mosquana A, Hakim O, Ohad N. FIE and CURLY LEAF polycomb proteins interact in the regulation of homeobox gene expression during sporophyte development. *Plant J.* 2004;37:707–19.
70. Hay A, Tsiantis M. KNOX genes: versatile regulators of plant development and diversity. *Development.* 2010;137:3153–65.
71. Lodha M, Marco CF, Timmermans MCP. The ASYMMETRIC LEAVES complex maintains repression of KNOX homeobox genes via direct recruitment of Polycomb-repressive complex2. *Genes Dev.* 2013;27:596–601.
72. Eulgem T, Rushton PJ, Robatzek S, Somssich IE. The WRKY superfamily of plant transcription factors. *Trends Plant Sci.* 2000;5:199–206.
73. Chen LQ, Qu XQ, Hou BH, Sosso D, Osorio S, Fernie AR, et al. Sucrose efflux mediated by SWEET proteins as a key step for phloem transport. *Sci (80-).* 2012;335:207–11.
74. Artus NN, Uemura M, Steponkus PL, Gilmour SJ, Lin C, Thomashow MF. Constitutive expression of the cold-regulated Arabidopsis thaliana COR15a gene affects both chloroplast and protoplast freezing tolerance. *Proc Natl Acad Sci USA.* 1996;93:13404–9.
75. Thalhammer A, Bryant G, Sulpice R, Hinch DK. Disordered cold regulated15 proteins protect chloroplast membranes during freezing through binding and folding, but do not stabilize chloroplast enzymes in vivo. *Plant Physiol.* 2014;166:190–201.
76. Ciftci-Yilmaz S, Morsy MR, Song L, Coutu A, Krizek BA, Lewis MW, et al. The EAR-motif of the Cys2/His2-type zinc finger protein Zat7 plays a key role in the defense response of Arabidopsis to salinity stress. *J Biol Chem.* 2007;282:9260–8.
77. Le Hir R, Spinner L, Klemens PAW, Chakraborti D, de Marco F, Vilaine F, et al. Disruption of the sugar transporters AtSWEET11 and AtSWEET12 affects vascular development and freezing tolerance in Arabidopsis. *Mol Plant.* 2015;8:1687–90.

78. Rauluseviciute I, Riudavets-Puig R, Blanc-Mathieu R, Castro-Mondragon JA, Ferenc K, Kumar V, et al. JASPAR 2024: 20th anniversary of the open-access database of transcription factor binding profiles. *Nucleic Acids Res.* 2024;52:D174–82.
79. Zhao J, Favero DS, Qiu J, Roalson EH, Neff MM. Insights into the evolution and diversification of the AT-hook Motif Nuclear Localized gene family in land plants. *BMC Plant Biol.* 2014;14:266.
80. Andersen SU, Algreen-Petersen RG, Hoedl M, Jurkiewicz A, Cvitanich C, Braunschweig U, et al. The conserved cysteine-rich domain of a tesmin/TSO1-like protein binds zinc in vitro and TSO1 is required for both male and female fertility in *Arabidopsis thaliana*. *J Exp Bot.* 2007;58(13):3657–70.
81. Li Y, Yang Z, Zhang Y, Guo J, Liu L, Wang C, et al. The roles of HD-ZIP proteins in plant abiotic stress tolerance. *Front Plant Sci.* 2022;13:1027071.
82. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 2014;15:550.
83. Vyse K, Faivre L, Romich M, Pagter M, Schubert D, Hinch DK, et al. Transcriptional and post-transcriptional regulation and transcriptional memory of chromatin regulators in response to low temperature. *Front Plant Sci.* 2020;11:39.
84. Lu F, Cui X, Zhang S, Jenuwein T, Cao X. *Arabidopsis* REF6 is a histone H3 lysine 27 demethylase. *Nat Genet.* 2011;43:715–9.
85. Crevillén P, Yang H, Cui X, Greeff C, Trick M, Qiu Q, et al. Epigenetic reprogramming that prevents transgenerational inheritance of the vernalized state. *Nature.* 2014;515:587–90.
86. Lu F, Cui X, Zhang S, Liu C, Cao X. JMJ14 is an H3K4 demethylase regulating flowering time in *Arabidopsis*. *Cell Res.* 2010;20:387–90.
87. Deleris A, Greenberg MVC, Ausin I, Law RWY, Moissiard G, Schubert D, et al. Involvement of a Jumonji-C domain-containing histone demethylase in DRM2-mediated maintenance of DNA methylation. *EMBO Rep.* 2010;11:950–5.
88. Yoshida N, Yanai Y, Chen L, Kato Y, Hiratsuka J, Miwa T, et al. EMBRYONIC FLOWER2, a novel Polycomb group protein homolog, mediates shoot development and flowering in *Arabidopsis*. *Plant Cell.* 2001;13:2471–81.
89. Gendall AR, Levy YY, Wilson A, Dean C. The VERNALIZATION 2 gene mediates the epigenetic regulation of vernalization in *Arabidopsis*. *Cell.* 2001;107:525–35.
90. Turck F, Roudier F, Farrona S, Martin-Magniette ML, Guillaume E, Buisine N, et al. *Arabidopsis* TFL2/LHP1 specifically associates with genes marked by trimethylation of histone H3 lysine 27. *PLoS Genet.* 2007;3:e86.
91. Beine-Golovchuk O, Firmino AAP, Dąbrowska A, Schmidt S, Erban A, Walther D, et al. Plant temperature acclimation and growth rely on cytosolic ribosome biogenesis factor homologs. *Plant Physiol.* 2018;176:2251–76.
92. Cheong BE, Beine-Golovchuk O, Gorka M, Ho WWH, Martinez-Seidel F, Firmino AAP, et al. *Arabidopsis* REI-LIKE proteins activate ribosome biogenesis during cold acclimation. *Sci Rep.* 2021;11:2410.

Figures

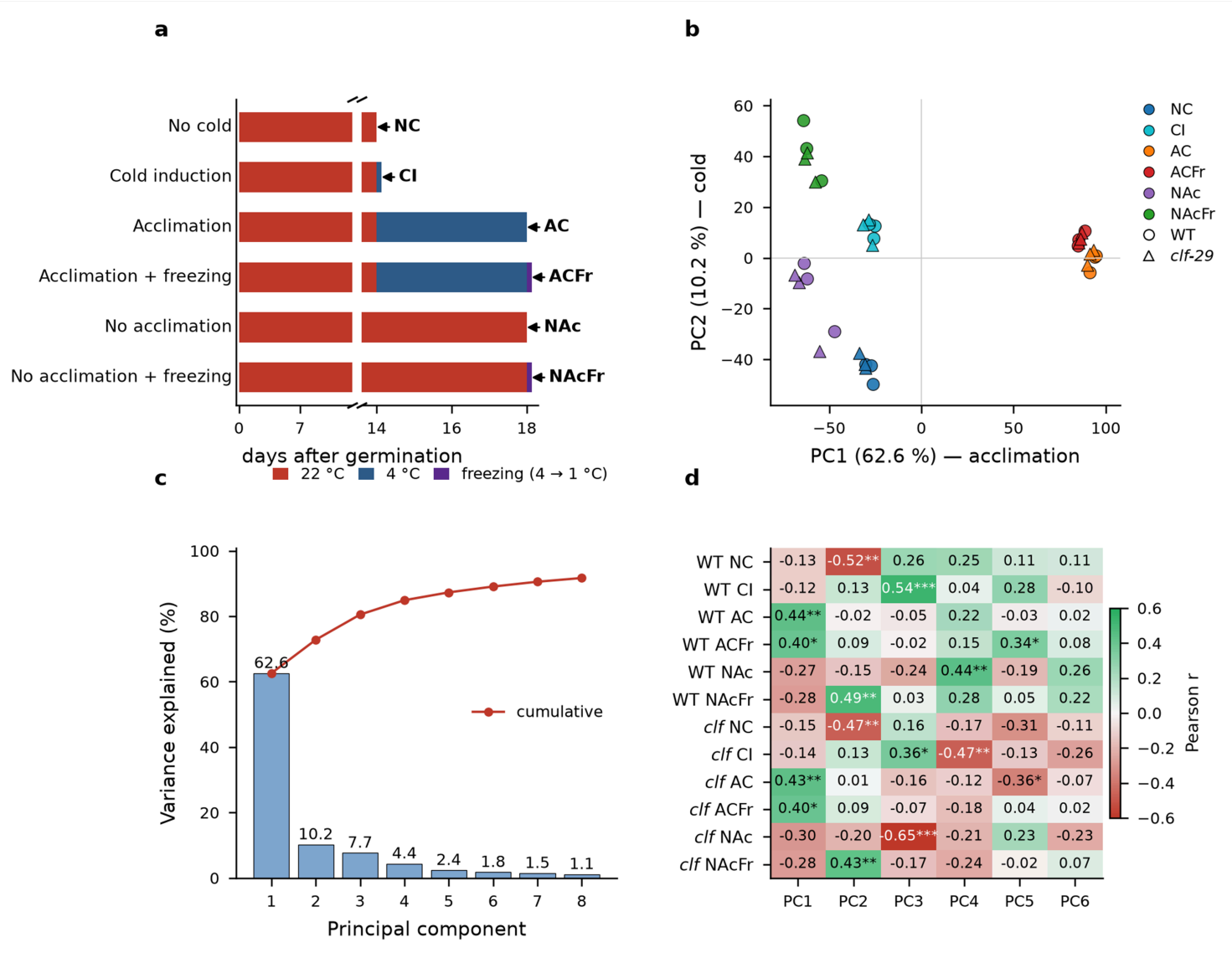


Figure 1

Global transcriptomic dynamics of the cold time course in WT and *clf-29*. (a) Diagram of the RNA-seq experimental set-up, with the temperature regime on a time axis: growth at 22 °C, cold induction for 3 h at 4 °C, acclimation at 4 °C for 4 d with an age-matched non-acclimated control at 18 d, and freezing applied as a 3 h ramp from 4 °C to 1 °C in the dark. (b) Principal component analysis of the 36 samples (PC1 vs PC2), with condition indicated by colour and genotype by symbol (circles, WT; triangles, *clf-29*). (c) Scree plot of the variance explained by each component, with the cumulative curve. (d) Correlation of each principal component with each genotype-by-condition group, each group being coded as an indicator variable against all other samples (Pearson *r*; * *p*<0.05, ** *p*<0.01, *** *p*<0.001). The orientation

of a principal component is arbitrary; PC4, PC5 and PC6 are drawn with the sign shown here. VST-normalised data (blind mode), 21,285 genes, 36 samples.

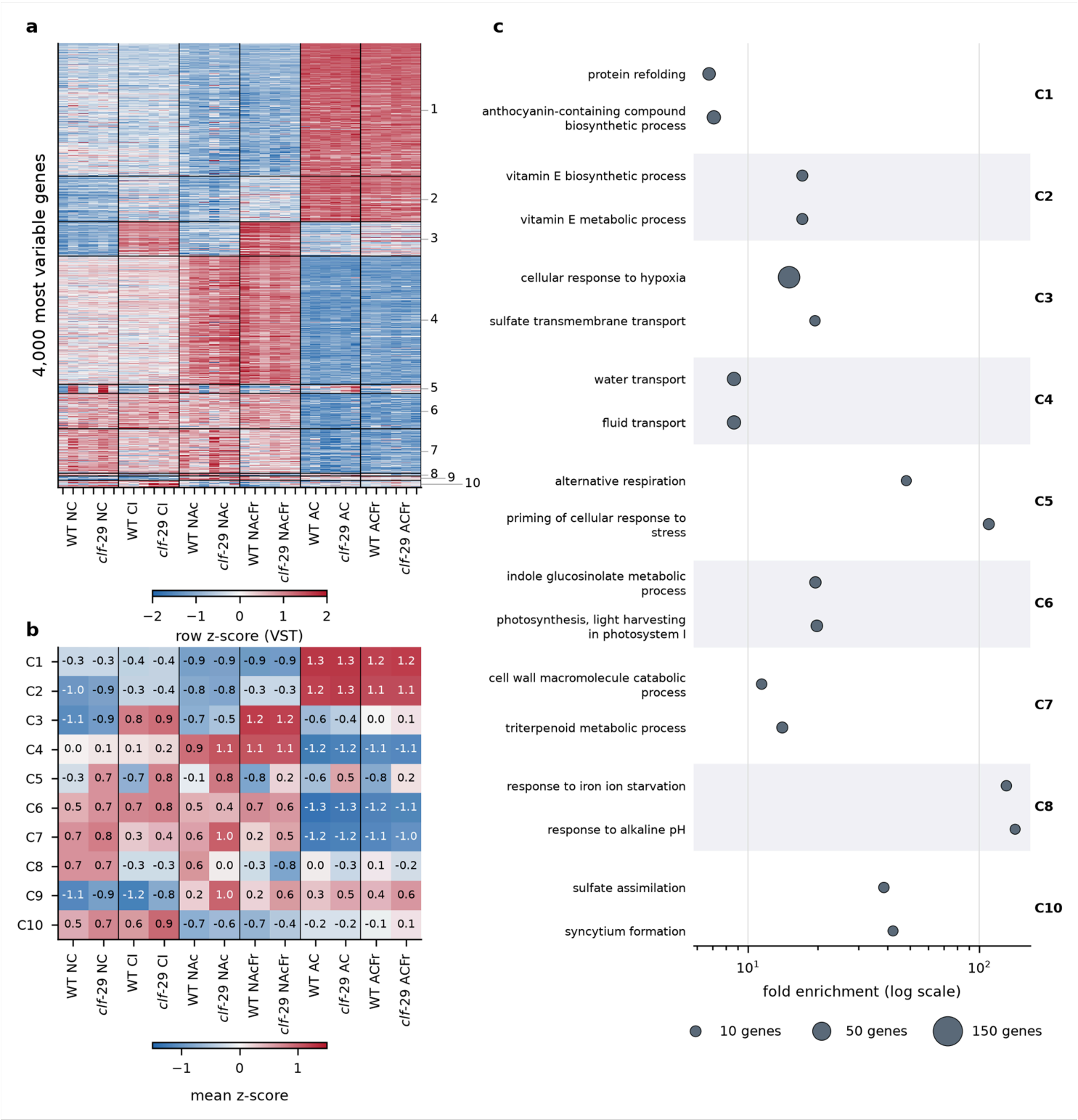


Figure 2

Global expression structure of the cold time course and the biological processes behind it. (a) Heatmap of the 4,000 most variable genes, partitioned into ten clusters by hierarchical clustering of the row z-scores

of the variance-stabilised counts; colour scale saturating at ± 2 , cluster number on the right. (b) Mean profile of each cluster within each genotype and condition (scale ± 1.5). (c) Gene Ontology of Biological Process for each cluster, tested against the 21,285 expressed genes; the two most enriched terms per cluster are shown among those with an adjusted p value below 0.05 (Benjamini-Hochberg). Dot position is fold enrichment on a logarithmic axis shared by all clusters, dot area the number of genes annotated to the term; cluster 9 has no significant term and is not shown. All significant terms are in Table S1. In a and b, conditions are ordered NC, CI, NAc, NAcFr, AC, ACFr, with three WT replicates followed by three *c/f*-29 replicates.

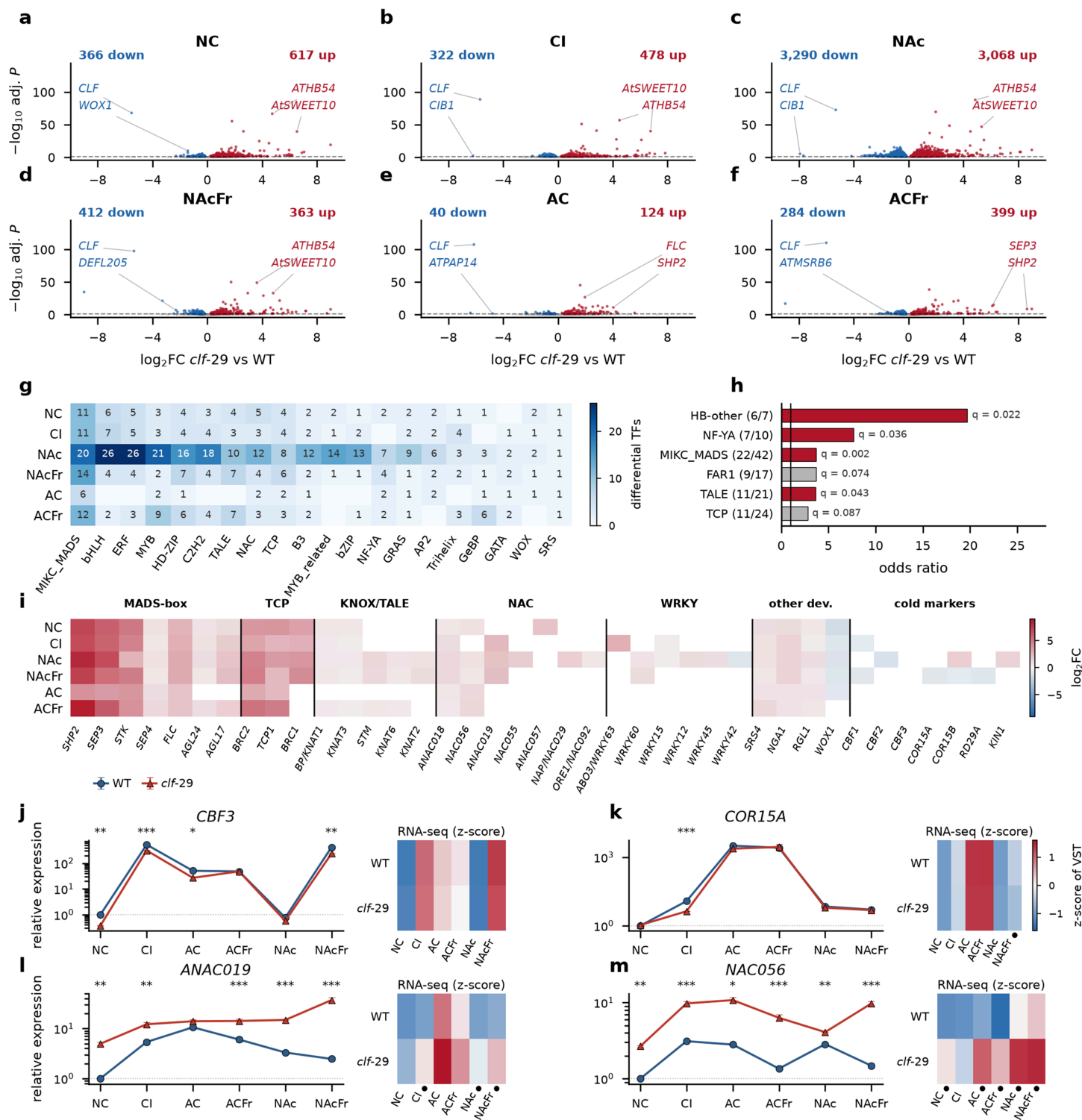


Figure 3

Transcriptional consequences of the lack of CLF across the six conditions. (a-f) Volcano plots of *clf-29* versus WT at NC, CI, NAc, NAcFr, AC and ACFr; red, up in the mutant; blue, down (adjusted $p < 0.05$); grey, not significant; the two most extreme named genes per side are labelled, with CLF as internal control. (g) Differentially expressed transcription factors (TFs) per family and condition. (h) Families enriched among the 405 CLF-dependent TFs (one-sided Fisher's exact test against 1,717 annotated

Arabidopsis TFs, Benjamini-Hochberg; red, $q < 0.05$). (i) log2 fold change (FC) of selected genes by family; blank, adjusted $p > 0.05$. (j-m) RT-qPCR of *CBF3*, *COR15A*, *ANAC019* and *NAC056* in one representative biological replicate of two (mean $\pm$ SD of the technical replicates, $\Delta\Delta C_t$ with *ACT2* as reference, non-acclimated WT as calibrator); asterisks, Welch's t-test p-value on the ΔC_t of the technical replicates: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; values in Table S5. Right, z-score of the variance-stabilised values; ●, significant in the transcriptomic analysis (adjusted $p < 0.05$).

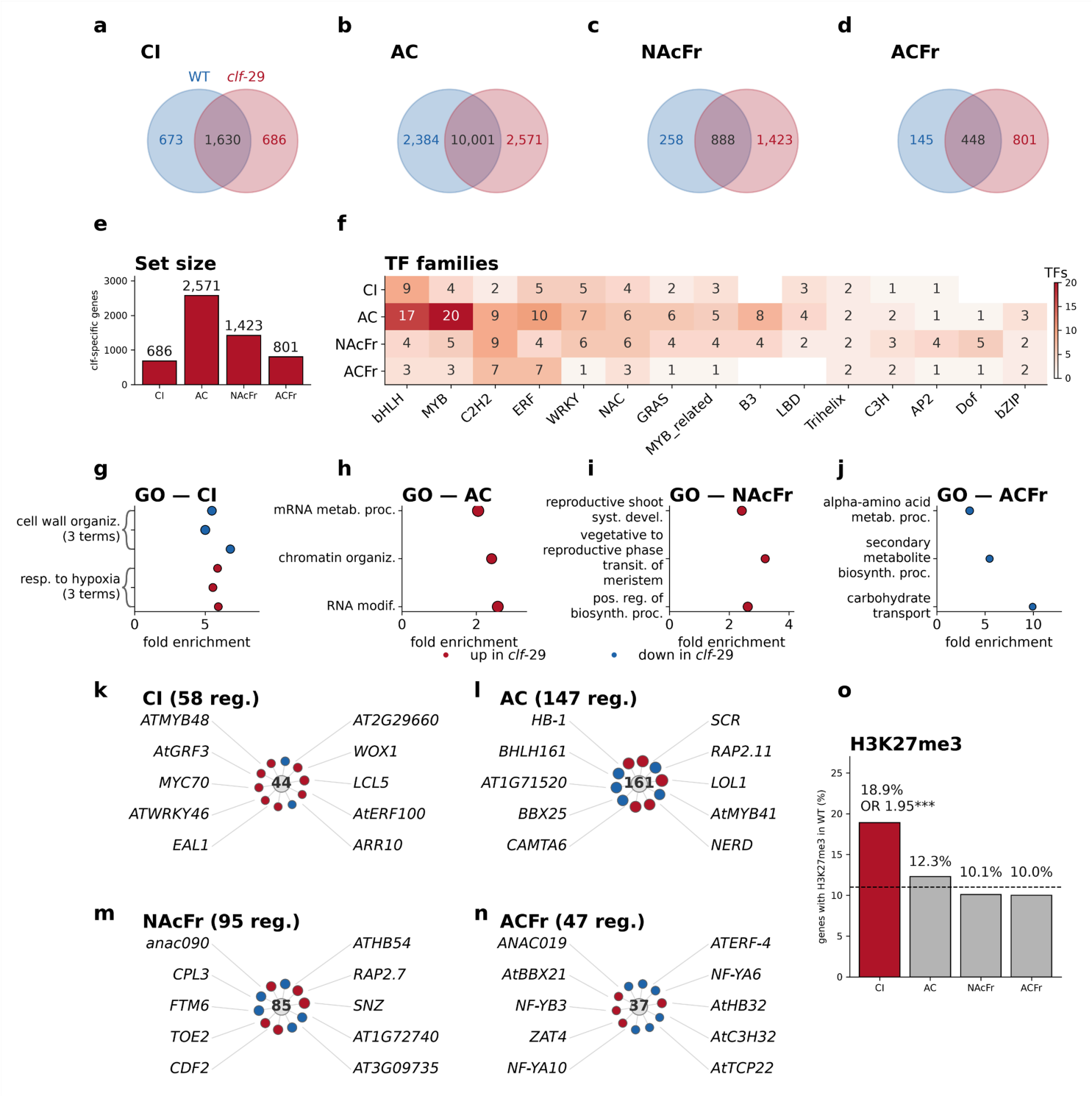


Figure 4

***clf*-specific gene sets: regulators, functions and Polycomb marking.** (a–d) Overlap between the genes responding to each temperature shift in the WT and in *clf-29*, for CI, AC, NAcFr and ACFr; the *clf*-specific set is the mutant-only fraction. (e) Size of the four *clf*-specific sets. (f) Differentially expressed transcription factors per family and condition. (g–j) Gene Ontology terms enriched in each set (adjusted $p < 0.01$; three per direction), with dot area proportional to gene number and colour indicating the direction of change in *clf-29*. Term names are abbreviated and terms of the same branch are bracketed and labelled once; full names and statistics are in Table S6. (k–n) The ten regulators with the largest summed edge weight in each set, coloured by direction of change; the central node is the number of targets shared by two or more of them (44, 161, 85 and 37 of 133, 485, 232 and 151 targets). (o) Percentage of genes carrying H3K27me3 in WT chromatin (37) dashed line, genome background (11.0%) over the 20,689 expressed genes with annotated coordinates; two-sided Fisher's exact test, Benjamini-Hochberg correction. Only the cold-induction set is enriched (18.9%, OR 1.95, adjusted $p < 0.001$); the other three are at background level (OR 1.16, 0.90 and 0.90, not significant).

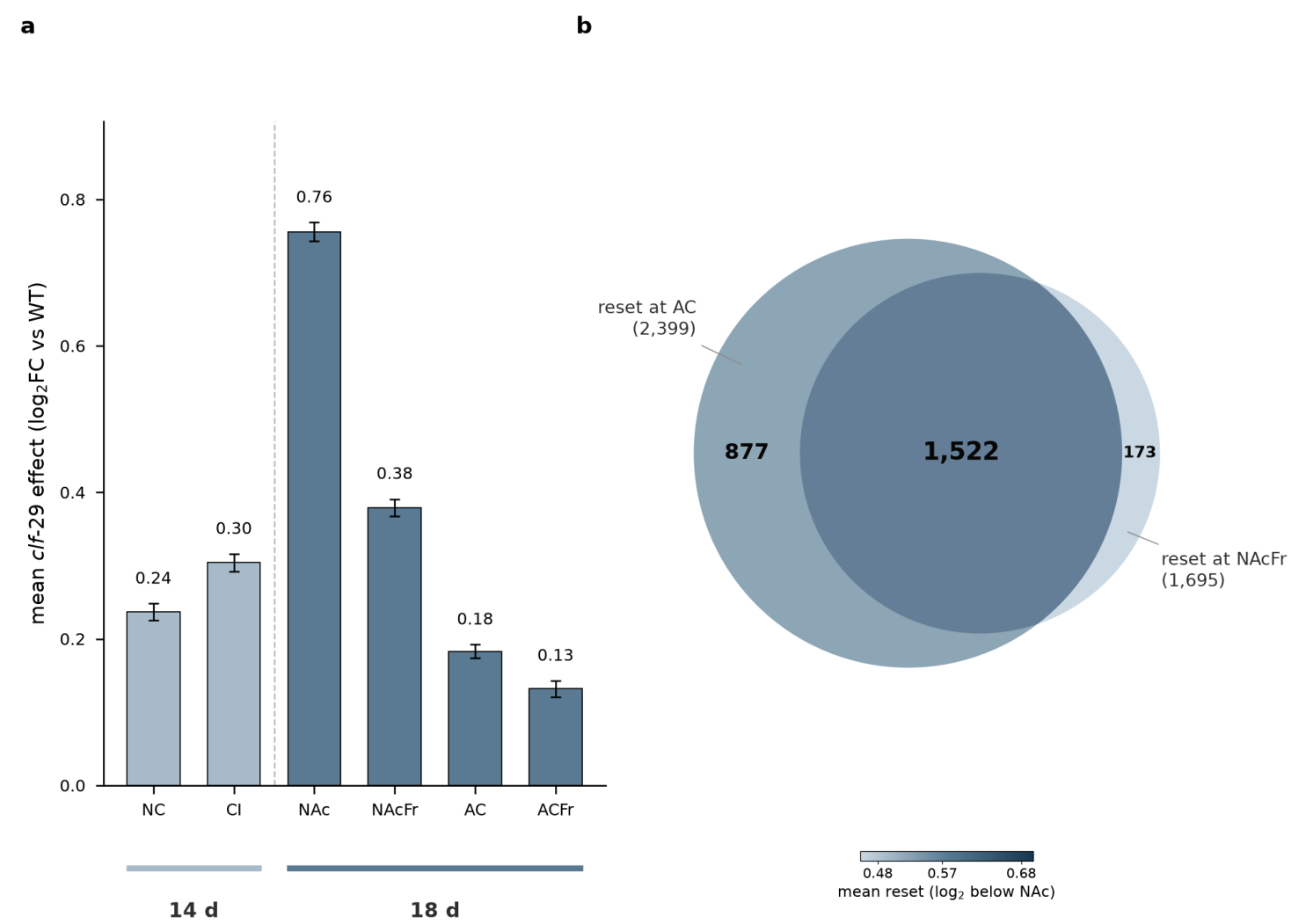


Figure 5

clf-dependent derepression is attenuated by cold. (a) Mean *clf-29* versus WT effect (log2 fold change) on the 3,068 genes up-regulated in the mutant at NAc, across all six conditions; bars are coloured by plant age (light, 14 d; dark, 18 d) and error bars show SEM. The effect peaks at NAc (0.76) and falls to 0.18 after acclimation and 0.13 after freezing following acclimation. (b) Overlap between the genes reset at acclimation (2,399) and those reset by freezing without acclimation (1,695); a gene was scored as reset when its *clf-29* versus WT effect decreased by more than 0.3 log2 units relative to NAc. Circle areas are proportional to set size and the area of overlap to the 1,522 shared genes; each region is filled according to the mean reset of its genes (colour bar, log2 units below NAc), the overlap being the superposition of the two fills.

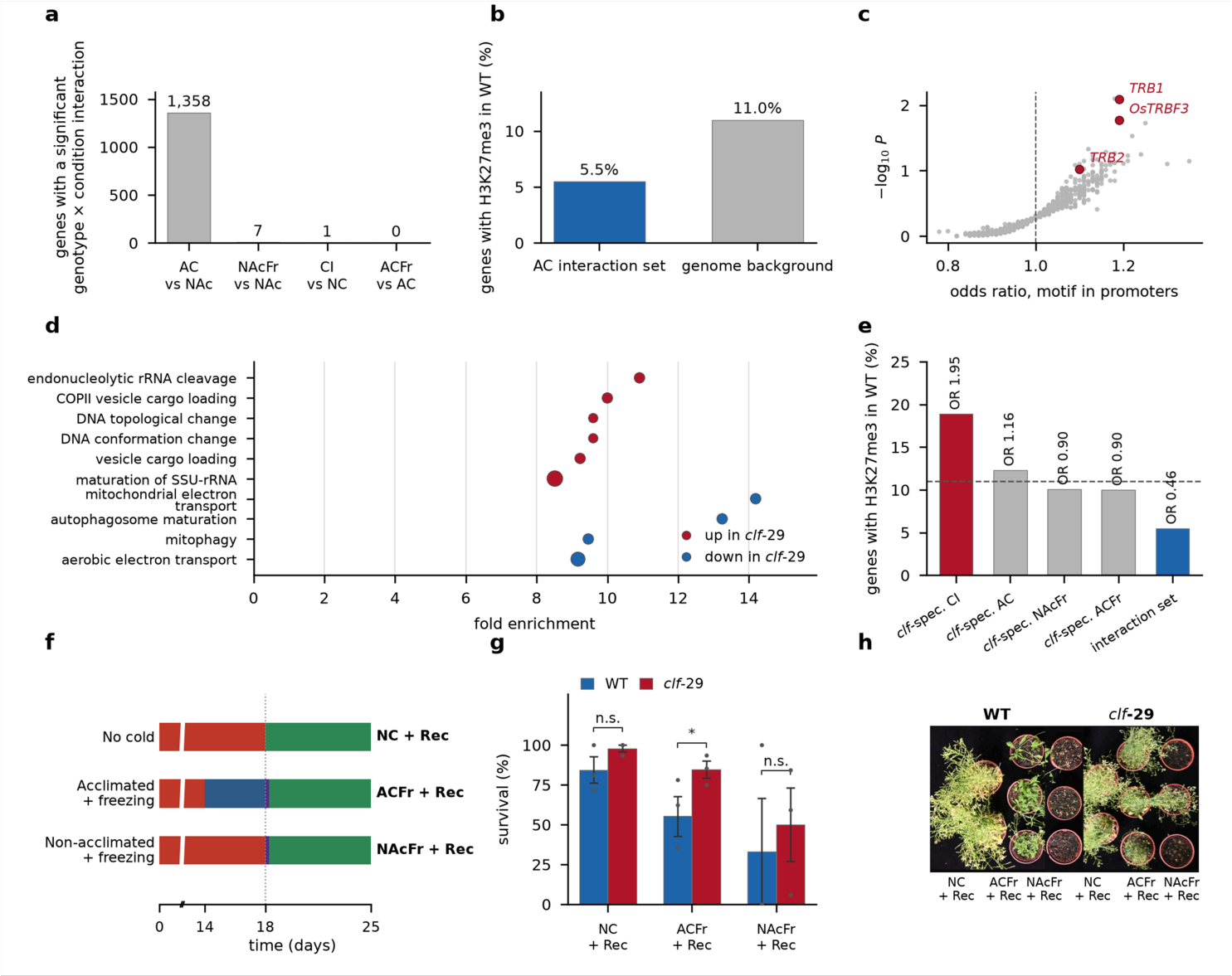


Figure 6

CLF-dependent remodelling is confined to acclimation, and *clf-29* survives freezing better than the WT. (a) Genes with a significant genotype × condition interaction term at each cold transition (DESeq2, adjusted $p < 0.05$). (b) Percentage of the 1,325 interaction genes with annotated coordinates, out of the 1,358

detected, carrying H3K27me3 in WT chromatin (37), next to the genome background: 5.5 % versus 11.0 %, odds ratio 0.46, $p= 1.3 \times 10^{-12}$ (two-sided Fisher's exact test). (c) Odds ratio against uncorrected p for the 703 JASPAR matrices tested in their promoters, the three telobox/TRB matrices highlighted.(d) GO enrichment of the same genes in both directions (94 terms up and 23 down in *clf-29* at adjusted $p< 0.05$); six up and four down terms shown, dot area proportional to the genes in the term, full lists in Table S11. (e) The same measurement on the four *clf*-specific sets and on the interaction set; numbers above the bars are odds ratios, dashed line, genome background (11.0 % over the 20,689 expressed genes with annotated coordinates; two-sided Fisher's exact test, Benjamini-Hochberg correction). (f) Set-up of the freezing assay, adapted from (42): 16 plants per pot, six to seven pots per genotype and regime, three biological replicates; $-7\text{ }^{\circ}\text{C}$ for 6 h and 7 d of recovery before scoring. Time axis broken during growth. Regimes are labelled as in the RNA-seq (NC, ACFr, NAcFr); + Rec marks scoring after the 7-d recovery. (g) Survival of the WT and *clf-29*; bars, percentage of all plants (96 to 112 per group); error bars, standard error across the three biological replicates; points, replicate means *, $p < 0.05$ in a Welch's t test within regime. (h) A representative biological replicate at the end of recovery; WT left, *clf-29* right.



Figure 7

The Polycomb machinery and the CBF regulon in *clf-29*. (a) Cold response of the H3K27me3 writers, erasers and readers at each of the four cold transitions, expressed as the difference of differences: the response of each gene in *clf-29* minus its response in the WT, so a positive value means the gene responds more to cold when CLF is absent. Colour spans ± 0.72 on the same scale (blue, weaker; red, stronger response in the mutant); every cell carries its value. *, $p < 0.05$ in a Student's *t* test on the replicate-level differences. (b) The same values at the acclimation transition, ranked; *p* values and test as in (a). (c-f) Expression of *CBF1*, *CBF2*, *CBF3* and *COR15A* across the six conditions in both genotypes (variance-stabilised counts; mean of three biological replicates $\pm$ standard error, replicates shown as faint points). The three *CBF* panels share a common scale.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Additionalfile1TableS1.xlsx](#)
- [Additionalfile2TableS2.xlsx](#)
- [Additionalfile7FigureS2.png](#)
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- [Additionalfile10FigureS3.pdf](#)
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