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SVALKA-POLYCOMB REPRESSIVE COMPLEX2 module controls *C-REPEAT BINDING FACTOR3* induction during cold acclimation

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Abstract

C-REPEAT BINDING FACTORS (CBFs) are highly conserved plant transcription factors that promote cold tolerance. In Arabidopsis (*Arabidopsis thaliana*), three CBFs (CBF1 to CBF3) play a critical role in cold acclimation, and the expression of their corresponding genes is rapidly and transiently induced during this adaptive response. Cold induction of *CBFs* has been extensively studied and shown to be tightly controlled, yet the molecular mechanisms that restrict the expression of each *CBF* after their induction during cold acclimation are poorly understood. Here, we present genetic and molecular evidence that the decline in the induction of *CBF3* during cold acclimation is epigenetically regulated through the Polycomb Repressive Complex (PRC) 2. We show that this complex promotes the deposition of the repressive mark H3K27me3 at the coding region of *CBF3*, silencing its expression. Our results indicate that the cold-inducible long noncoding RNA *SVALKA* is essential for this regulation by recruiting PRC2 to *CBF3*. These findings unveil a *SVALKA*-PRC2 regulatory module that ensures the precise timing of *CBF3* induction during cold acclimation and the correct development of this adaptive response.

Introduction

Low temperatures adversely affect the growth and development of plants, and condition their geographic distribution and productivity. To cope with low temperatures and acquire freezing tolerance, plants from temperate regions have evolved a sophisticated adaptive response, termed cold acclimation, whereby they increase their freezing tolerance after being exposed to low nonfreezing temperatures ([Thomashow 1999](#)). In Arabidopsis (*Arabidopsis thaliana*),

crucial to this response are the *C-REPEAT BINDING FACTORS* (*CBFs*), a small family of three tandemly clustered cold-inducible genes (*CBF1* to *CBF3*) encoding transcription factors that activate the expression of different cold-regulated (*COR*) genes, known as the CBF regulon, to boost freezing tolerance ([Gilmour et al. 1998](#); [Jaglo-Ottosen et al. 1998](#); [Liu et al. 1998](#); [Medina et al. 1999](#); [Novillo et al. 2007](#)). The expression of *CBFs* during cold acclimation is characterized by a transient induction with a peak within 3 to 6 h of cold exposure and a

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subsequent rapid decline, after 8 to 12 h of exposition, to attain a relatively stable level (Gilmour et al. 1998; Liu et al. 1998; Medina et al. 1999). Despite extensive studies on the regulation of *CBF* cold induction, the molecular mechanisms underlying the posterior decline of expression remain unclear. Here, we show that the Arabidopsis cold-inducible long noncoding RNA (lncRNA) *SVALKA*, which is transcribed on the antisense strand between *CBF3* and *CBF1* (Kindgren et al. 2018), recruits the Polycomb Repressive Complex (PRC) 2 to the *CBF3* gene for deposition of the H3K27me3 repressive mark, thus restraining its expression after its induction peak and ensuring the correct development of the cold acclimation process. Our study reveals that the expression of *CBFs* during cold acclimation is epigenetically regulated through a *SVALKA*-PRC2 module and this regulation is essential for this adaptive response.

Results and discussion

Under control conditions, CURLY LEAF (CLF), an essential subunit of the Arabidopsis PRC2 complex, has been associated to the genomic region harboring the *CBFs* (Xiao et al. 2017), and, consistently, the PRC2-dependent repressive mark H3K27me3 is substantially deposited at that region (Sequeira-Mendes et al. 2014). We then decided to explore the possibility that the PRC2 might be involved in repressing the expression of *CBFs* after their induction during cold acclimation. To test this possibility, we assessed the expression of *CBFs* in Arabidopsis *clf28 swn7* mutants exposed 3 or 24 h to 4 °C, times at which they are highly induced or their induction has already drastically decreased in Col-0 wild-type (WT) plants, respectively (Supplementary Fig. S1). The *clf28 swn7* mutants have a compromised PRC2 activity due to null mutations in *CLF* and *SWN* genes, which encode two of its core subunits, giving rise to small seedlings after germination that degenerate to a callus-embryo-like structure (Farrona et al. 2011). Reverse transcription quantitative PCR (RT-qPCR) assays with specific primers for each *CBF* (Fig. 1A, Supplementary Table S1) revealed that *CBF1* and *CBF3* transcripts accumulated at higher levels in mutant than in the WT plants in response to low temperature, this accumulation being much more significant after 24 h of cold exposure (Fig. 1B). The transcript levels of *CBF2*, however, were not affected in the mutants (Fig. 1B). These data indicated that PRC2 suppresses the expression of *CBF1* and *CBF3*, mainly in the late phase of their induction by low temperature. The induction of *CBF2* does not seem to be silenced by PRC2. Despite their redundant function in cold acclimation, the expression of *CBF* genes is positively and negatively governed through a plethora of common and specific regulators (Ding et al. 2020). Therefore, it is not surprising that PRC2 only silences *CBF1* and *CBF3*. Understanding how *CBF2* gene expression is repressed once it is induced under low temperature conditions requires additional experiments and will be the subject of future studies.

From the results described above, we anticipated that, after 24 h of cold exposure, CLF would be substantially associated to *CBF1* and *CBF3* chromatin. This assumption was examined by determining the levels of CLF associated to *CBFs* by means of chromatin immunoprecipitation (ChIP) assays, using an anti-MYC antibody, chromatin isolated from the WT transgenic plants expressing a translational *35S:MYC:CLF* fusion (Lodha et al. 2013) grown under control conditions or exposed to 4 °C for 24 h, and specific primers corresponding to different regions of the *CBFs* (Fig. 1A, Supplementary Table S1). Results showed that CLF was significantly associated to the *CBF3* coding-region chromatin after 24 h of cold exposure. The levels of CLF associated to the chromatin upstream of the *CBF3* coding region also resulted to be significantly high after 24 h at 4 °C, although to a lower extent than those detected in the chromatin of the coding region. We did not find a significant association of CLF to the chromatin downstream of the *CBF3* coding region (Fig. 1C). Intriguingly, the levels of CLF associated with *CBF1* chromatin were not significant in any of the regions analyzed neither in plants grown under control conditions nor exposed to low temperature (Fig. 1C). Consistent with the data described above (Fig. 1B), the CLF levels associated with *CBF2* chromatin were not significant either (Fig. 1C). Then, we carried out ChIP assays with an anti-H3K27me3 antibody, chromatin from the WT plants grown under control conditions or subjected to 4 °C for 24 h, and the *CBF*-related primers used in the previous experiment (Fig. 1C, Supplementary Table S1). Under control conditions, coherently with the low expression of *CBF3*, the levels of the repressive histone mark H3K27me3 were highly significant in its chromatin corresponding to both promoter and coding regions (Fig. 1D). Although these regions showed very low levels of associated CLF (Fig. 1C), this is not surprising considering that around three quarters of the H3K27me3-marked genes have been proposed to not display CLF associated with their chromatin (Shu et al. 2019). These marks would have been deposited by a PRC2 containing a different catalytic subunit than CLF, such as SWN7 or MEA, or in a previous developmental stage (Costa and Dean 2019). Interestingly, after low temperature exposition, the levels of the H3K27me3 histone mark were significantly higher only in the *CBF3* coding-region chromatin (Fig. 1D). Altogether, these results strongly suggested that the PRC2 complex represses the expression of *CBF3* during cold acclimation, once attained its induction peak, through the deposition of the H3K27me3 mark at its coding region. The PRC2 complex would also suppress the expression of *CBF1* during cold acclimation but independently of CLF, through another component of the PRC2 like SWN, or through an indirect pathway.

Now, the arising question was how the PRC2 complex is associated to the chromatin of *CBF3* during cold acclimation. lncRNAs have recently emerged as potent regulators of gene expression and are recognized as important participants in PRC2 function (Margueron and Reinberg 2011). In Arabidopsis, *COLDAIR*, *COLDWRAP*, and *AG-lncRNA4*
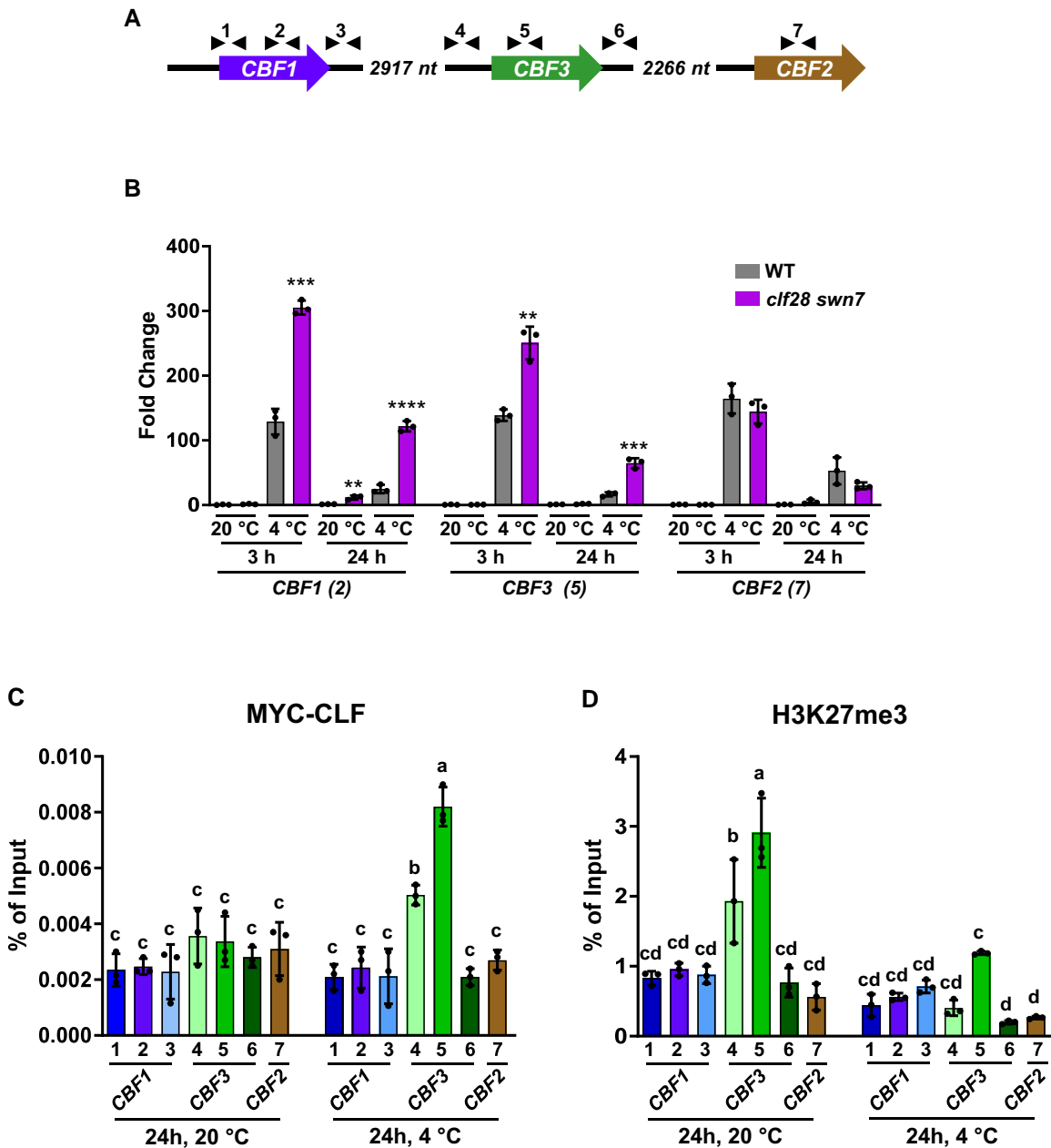
Figure 1. The cold induction of *CBF3* is repressed by the PRC2 complex. **A)** Schematic representation of the Arabidopsis genomic region containing the *CBF* genes. Arrowheads indicate the relative positions of primer sets used for expression analyses and ChIP experiments in this work. Each primer set is identified by a number. **B)** Expression analysis of *CBF1*, *CBF3*, and *CBF2* in 2-wk-old Col-0 (WT) and *clf28 swn7* plants maintained or exposed to 20 or 4 °C for 3 or 24 additional hours, respectively. In each condition (20 or 4 °C), transcript levels, determined by RT-qPCR, are represented as relative to the values of the corresponding WT. Asterisks indicate significant differences (**$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$) between *clf28 swn7* and WT in each condition, as determined by two-sided *t* test. The numbers in parentheses indicate the primer sets used. **C** and **D)** ChIP experiments showing the levels of MYC-CLF **(C)** and H3K27me3 **(D)** on the chromatin of *CBFs* from 2-wk-old WT plants grown 24 additional hours at 20 or 4 °C. The relative amounts of DNA in the input and the immunoprecipitated samples were determined by qPCR with the *CBF* primer sets indicated below the bars in three biological replicates per sample. The levels of MYC-CLF and H3K27me3 were determined as the percentage of input DNAs recovered in the immunoprecipitates. Distinct letters indicate significant differences ($P < 0.05$) according to one-way ANOVA followed by Tukey's test. In **B, C** and **D)** data represent the mean of three independent experiments and error bars show the standard deviation.

lncRNAs have been described to physically interact with the PRC2 subunit CLF, contributing to its localization to the corresponding target genes and their subsequent silencing through H3K27me3 deposition (Swiezewski et al. 2009; Kim and Sung 2017; Wu et al. 2018). A common feature of all these lncRNAs is that they are transcribed from the genomic locus of their target genes (Swiezewski et al. 2009; Kim and Sung 2017; Wu et al. 2018). Interestingly, as mentioned

above, the Arabidopsis genome encodes a cold-induced lncRNA, *SVALKA*, that is transcribed on the antisense strand between *CBF3* and *CBF1*. The transcription takes place from different transcription starting sites, originating short (α) and long (β) isoforms (Kindgren et al. 2018) (Supplementary Fig. S2A). Under control conditions, extensive transcription of the *SVALKA* β isoform throughout the *CBF1* gene body generates a natural antisense transcript that negatively regulates *CBF1* transcription (Zacharaki et al. 2023). In response to low temperature, the transcription of *SVALKA* isoforms is induced, reaching a stable maximum of induction after 8 to 12 h of treatment (Kindgren et al. 2018). It has been reported that a transcriptional read-through of the *SVALKA* β isoform limits the cold induction of *CBF1* by means of a head-to-head RNA polymerase II (RNAPII) collision mechanism at the 3′-end of this gene, negatively regulating cold acclimation (Kindgren et al. 2018). A function for the *SVALKA* isoforms, however, has not yet been described. Thus, we hypothesized that they could mediate the decline of *CBF3* expression that takes place after its rapid induction during cold acclimation by recruiting PRC2 to *CBF3*. This possibility was assessed by studying the expression levels of *CBF3* in a *SVALKA* T-DNA null mutant (*svk-1*) (Kindgren et al. 2018) exposed to 4 °C for different times. We did not find significant differences in *CBF3* transcript levels between mutant and the WT plants after 3 or 8 h of cold exposure. However, *CBF3* transcripts were significantly higher in *svk-1* than in the WT plants after being exposed to 4 °C for 24 or 72 h (Fig. 2A). Very similar results were obtained when analyzing the *SVALKA* T-DNA mutant *SVK OE*, which overexpresses a partial and aberrant *SVALKA* transcript (Kindgren et al. 2018) (Fig. 2A). Our results, therefore, unveiled a role for *SVALKA* consisting in promoting the decline of *CBF3* expression that takes place after its induction during cold acclimation. It is worth mentioning that, as displayed in Fig. 1B, *clf28 swn7* mutations lead to increased expression of *CBF3* at both 3 and 24 h after cold treatment, but disruption of *SVALKA* only affects *CBF3* expression after 24 h of exposure at 4 °C (Fig. 2A). Considering that *SVALKA* is not expressed after 3 h of cold treatment (Kindgren et al. 2018), we can conclude that the effect of PRC2 on *CBF3* expression in the early stage of cold acclimation is not mediated by *SVALKA*.

As shown above (Fig. 2A), *svk-1* and *SVK OE* mutants had in common a delay in the decline of *CBF3* induction during cold acclimation compared to the WT plants. Interestingly, however, they have been described to diverge in the induction levels of *CBF1*. Indeed, while the cold induction of *CBF1* is increased in *svk-1*, it is significantly reduced in *SVK OE* (Kindgren et al. 2018). The *SVK OE* mutant, therefore, constitutes a very good material to evaluate the physiological significance of the role unveiled for the *SVALKA* lncRNA. First, we determine the mRNA levels corresponding to some CBF-activated *COR* genes such as *KIN1*, *XERO2*, *GOLS3*, and *COR15B* in *SVK OE* plants subjected to low temperature for 24 and 72 h. *svk-1* mutants were also included in the analysis as a reference. Consistent with the high levels of *CBF3*

transcripts present in *SVK OE* and *svk-1* plants after a long-term cold treatment (Fig. 2A), the expression of *COR* genes was significantly higher in both mutants than in the WT plants exposed 72 h to 4 °C (Fig. 2B). Then, we studied the effect of the late up-regulation of *CBF3* and *COR* gene expression on the ability of *SVK OE* plants to tolerate freezing and cold acclimate. In this study, *svk-1* mutants were again included for reference. Two-wk-old nonacclimated and cold acclimated (seven additional days at 4 °C) mutant plants were subjected to decreasing freezing temperatures and, after 2 wk of recovery under control conditions, scored for survival. Nonacclimated mutants presented a similar capacity to tolerate freezing as the WT, the LT_{50} (temperature that causes 50% lethality) values being in both cases around −6.8 °C (Fig. 2C). By contrast, cold acclimated mutants exhibited a significantly higher freezing tolerance than cold acclimated WT plants, the LT_{50} values in this case being −10.8 °C for both *SVK OE* and *svk-1* mutants and −10.0 °C for the WT plants (Fig. 2D). As already mentioned, the expression of *CBF1* is only slightly induced by low temperature in the *SVK OE* mutant (Kindgren et al. 2018). Therefore, our findings clearly indicated that *SVALKA* also contributes to the correct development of cold acclimation by mediating the decline of *CBF3* induction during this adaptive response.

Next, we studied if *SVALKA* could contribute to suppress the induction of *CBF3* during cold acclimation by targeting PRC2 to this gene. First, the possible interaction of *SVALKA* with CLF was analyzed by means of RNA immunoprecipitation (RIP) assays. We used null *clf* double mutant plants *clf50 clf16* expressing a *35S:GFP:CLF* fusion (Schubert et al. 2006) grown under control conditions or exposed to 4 °C for 8 h, a time at which the expression of *SVALKA* is highly induced (Kindgren et al. 2018), and an anti-GFP antibody. Plants WT expressing a *35S:GFP* fusion were used as a control for specificity. The presence of *SVALKA* transcripts in the immunoprecipitates was determined and quantified by RT-qPCR with specific primers (Supplementary Fig. S2A, Supplementary Table S1) that, under our experimental conditions, solely amplify the spliced β isoform (Supplementary Fig. S2B). We only detected a significant enrichment of this isoform in the immunoprecipitated fraction corresponding to samples from plants expressing the *35S:GFP:CLF* fusion exposed to 4 °C (Fig. 3A), indicating that, indeed, *SVALKA* physically interacts with CLF in response to low temperature. Then, we assessed whether the interaction of *SVALKA* with CLF was required for targeting this protein, and therefore PRC2, to the *CBF3* coding region. ChIP experiments were performed with an anti-MYC antibody, chromatin isolated from *svk-1*, *SVK OE* and the WT plants expressing the *35S:MYC:CLF* fusion grown under control conditions or exposed to 4 °C for 24 h, and the specific *CBF3* primers corresponding to its coding region (Fig. 1A, Supplementary Table S1). The levels of MYC:CLF associated to the chromatin of *CBF1* coding-region in WT, *svk-1* and *SVK OE* plants under control and cold conditions were also quantified as internal controls in the
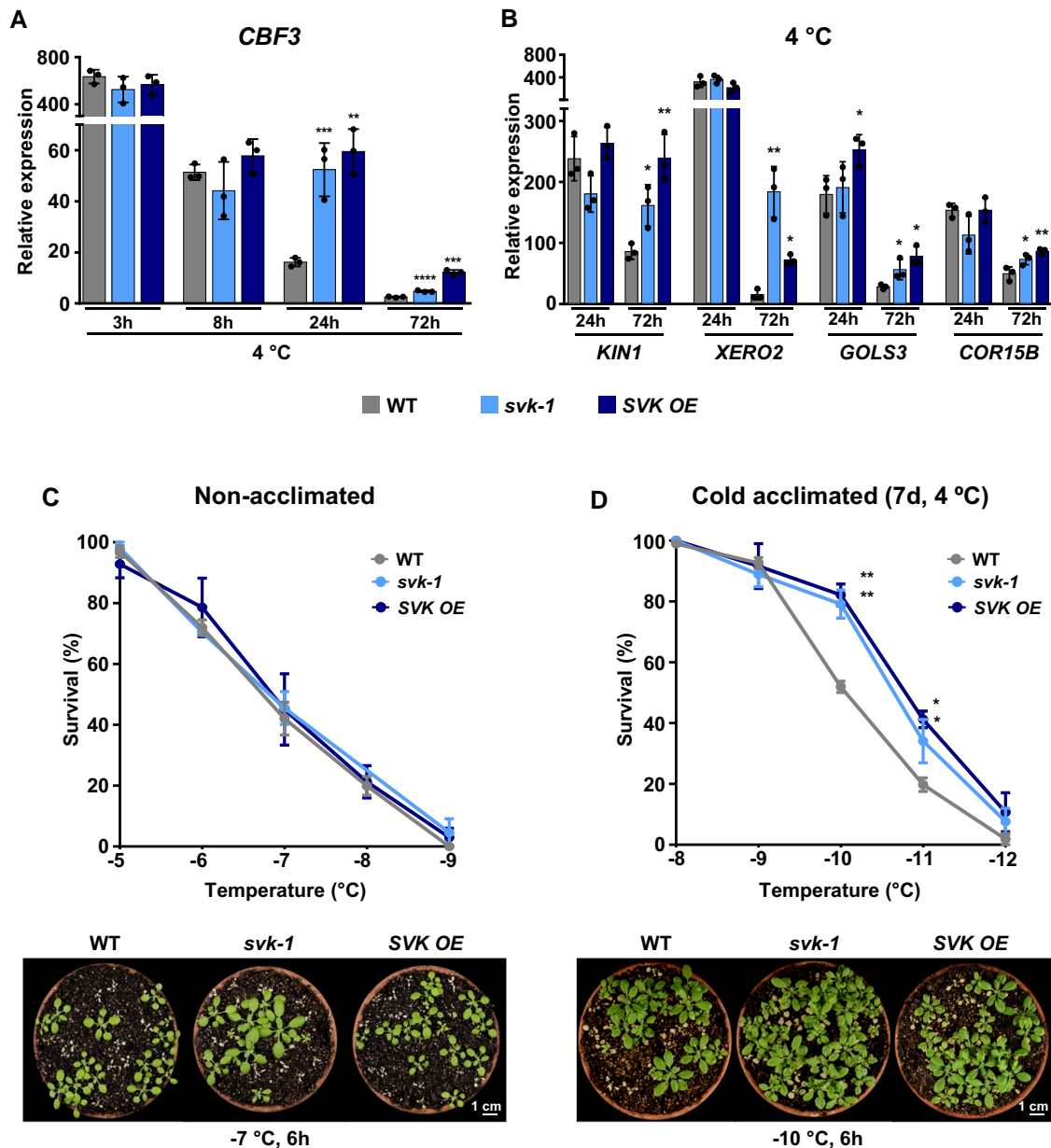
Figure 2. *SVALKA* regulates the cold acclimation response by mediating the decline of *CBF*3 induction. **A** and **B)** Expression analysis of *CBF3* (A) and CBF3 target genes *KIN1, XERO2, GOLS2,* and *COR15B* (B) in 2-wk-old WT, *svk-1* and *SVK OE* plants exposed to 4 °C for the indicated additional hours (3, 8, 24, or 72 h). In each case, transcript levels, determined by RT-qPCR, are represented as relative to the values of the WT plants grown under control conditions. Primer set 5 in Fig. 1A was used for expression analyses of *CBF3*. **C** and **D)** Freezing tolerance of nonacclimated **(C)** and cold acclimated 7 d at 4 °C **(D)** 2-wk-old WT, *svk-1* and *SVK OE* plants exposed to the indicated freezing temperatures for 6 h. Tolerance was estimated as the percentage of plants surviving each specific temperature after 1 wk of recovery under control conditions. Lower panels show the freezing tolerance of representative nonacclimated and cold acclimated plants. Images were digitally extracted for comparison. In all panels, asterisks indicate significant differences (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001) between *svk-1* and *SVK OE* mutants and the corresponding WT plants, as determined by one-sided *t* test. Data represent the mean of three independent experiments and error bars show the standard deviation.

experiments. The absence of a functional *SVALKA* in *svk-1* and *SVK OE* plants did not affect the levels of CLF associated with the *CBF3* chromatin in plants grown at 20 °C (Fig. 3B). After 24 h of cold exposure, however, the lack of a functional *SVALKA* led to significantly lower levels of associated CLF than in the WT plants, similar to those observed under control conditions (Fig. 3B). As expected, the levels of CLF

associated with the *CBF1* chromatin in *svk-1* and *SVK OE* mutants were, in all cases, as in the WT plants, which, in turn, were similar to those associated to *CBF3* chromatin in the WT plants grown under control conditions (Fig. 3B). These findings strongly supported that *SVALKA* interacts with CLF to promote PRC2 targeting to *CBF3* coding region after its induction during cold acclimation, and anticipated that
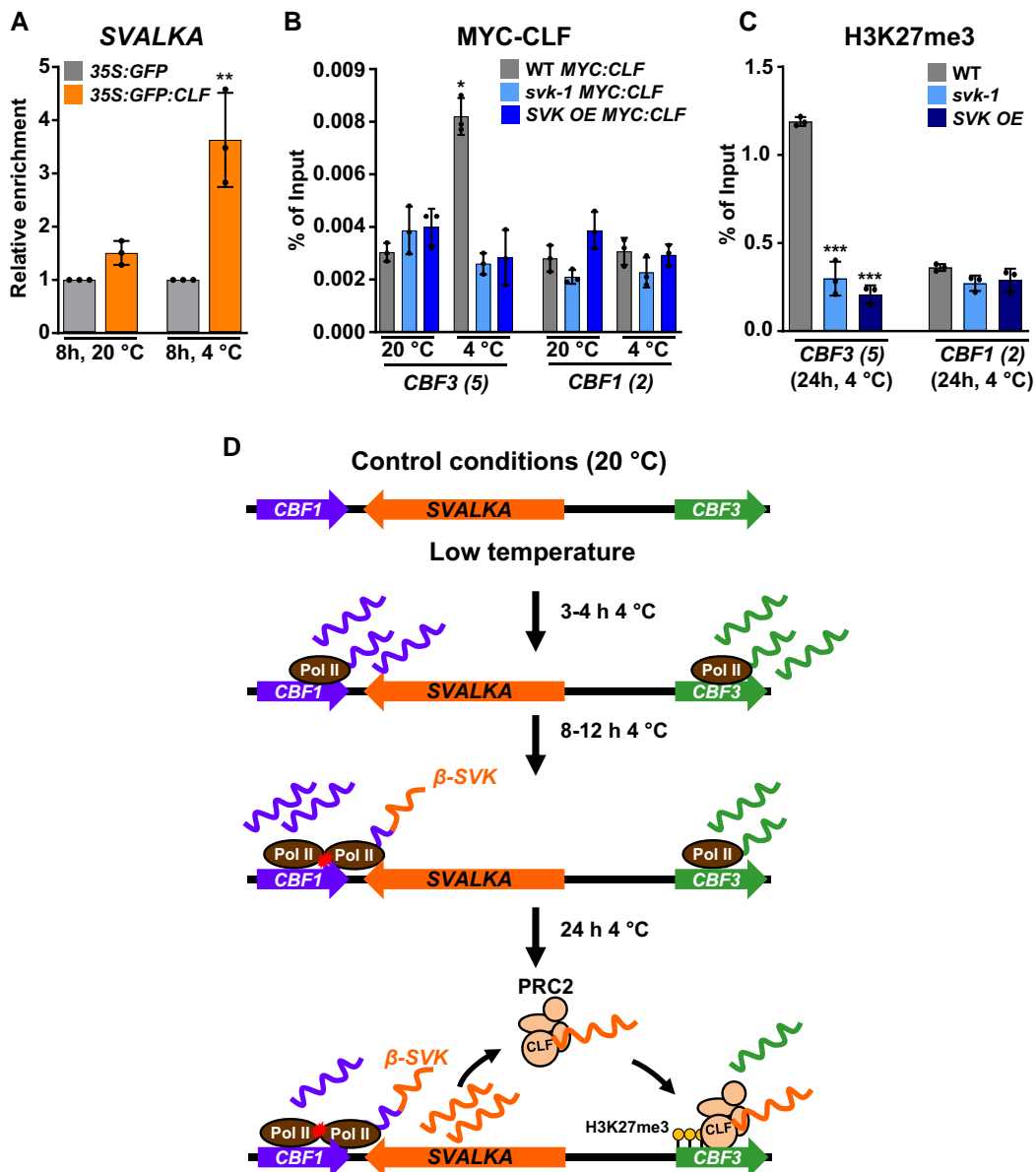
Figure 3. *SVALKA* interacts with CLF targeting PRC2 to *CBF3* for H3K27me3 deposition. **A)** RIP assays performed with 2-wk-old *clf50 clf16* plants expressing a *35S:GFP:CLF* fusion maintained or exposed to 20 or 4 °C for eight additional hours, respectively, and an anti-GFP antibody. Two-wk-old Col-0 (WT) plants expressing a *35S:GFP* fusion were used as interaction specificity controls. The relative amounts of *SVALKA* lncRNA (β isoform) in the input and the immunoprecipitated samples were determined by RT-qPCR, with three biological replicates per sample. The enrichment of *SVALKA* in the samples was determined as the percentage of input RNA recovered in the immunoprecipitates, and is represented relative to the enrichment in the corresponding *35S:GFP* samples. **B** and **C)** ChIP experiments showing the levels of MYC-CLF on the *CBF3* and *CBF1* chromatin of 2-wk-old WT, *svk-1* and *SVK OE* plants expressing the *MYC:CLF* fusion maintained at 20 °C or exposed 24 h to 4 °C **(B)**, and the levels of H3K27me3 on the *CBF3* and *CBF1* chromatin of 2-wk-old WT, *svk-1* and *SVK OE* plants exposed to 4 °C for 24 h **(C)**. The relative amounts of DNA in the input and the immunoprecipitated samples were determined by qPCR, with the *CBF* primer sets indicated in parenthesis (see Fig. 1A), in three biological replicates per sample. The levels of MYC-CLF and H3k27me3 were determined as the percentage of input DNA recovered in the immunoprecipitates. **D)** Proposed model for *SVALKA* function in Arabidopsis cold acclimation. In **(A)** and **(C)**, asterisks indicate significant differences (**$P < 0.01$, ***$P < 0.001$) between *35S:GFP:CLF* and *35S:GFP*, and *svk-1* or *SVK OE* and WT in *CBF3*, respectively, as determined by one-sided *t* test. In **(B)**, asterisk indicates significant differences (*$P < 0.05$) between *svk-1 MYC:CLF* or *SVK OE MYC:CLF* and WT *MYC:CLF* in *CBF3* at 20 °C, as determined by one-way ANOVA followed by Tukey's test. Data represent the mean of three independent experiments and error bars show the standard deviation.

SVALKA should be necessary for the deposition of the repressive histone mark H3K27me3 by PRC2 at the coding region of *CBF3*. This possibility was evaluated by quantifying the levels of H3K27me3 at the *CBF3* chromatin of WT, *svk-1* and *SVK* *OE* plants exposed to 4 °C for 24 h. The levels of H3K27me3 at the chromatin of *CBF1* were also quantified in these plants as an internal control. ChIP assays using an anti-H3K27me3 antibody and specific primers for *CBF1* and

CBF3 coding regions (Fig. 1A, Supplementary Table S1) revealed that, in fact, the levels of H3K27me3 at *CBF3* were significantly lower in both *svk-1* and *SVK OE* mutants than in the WT plants (Fig. 3C). Consistent with the results described above (Fig. 3B), the levels of H3K27me3 at *CBF1* were similar in all genotypes (Fig. 3C). All in all, these data indicated that *SVALKA* physically interacts with CLF to target PRC2 to *CBF3* coding region for deposition of H3K27me3 marks and repression of its induction during cold acclimation. As already proposed for different lncRNAs (Mattick et al. 2023), the specific recognition of the *CBF3* coding region by PRC2 for deposition of the H3K27me3 mark could lay on the capacity of *SVALKA* to recognize and interact with a *CBF3* specific nucleotide sequence, or with specific proteins associated to the *CBF3* coding region chromatin.

Based on the results described here, a model for *SVALKA* function in Arabidopsis cold acclimation is proposed in Fig. 3D. In response to low temperature, the expression of *CBFs* is rapidly induced, after 15 to 30 min, reaching the highest levels at 3 to 4 h of cold exposure. Around this time, a transcriptional read-through of *SVALKA* is activated, which would trigger head-to-head RNAPII collision over the *CBF1* gene leading to a regulated limitation of *CBF1* cold-induced levels. The transcription of *SVALKA* increases gradually during cold acclimation till attaining a maximum of induction after 8 to 12 h of exposure to low temperature. Then, the *SVALKA* β isoform would recruit the PRC2 complex to the coding region of *CBF3* gene, promoting the deposition of the H3K27me3 repressive mark and, consequently, the decrease of its expression. As a result, after 24 h under low temperature conditions, the *CBF3* expression would decay until reaching relatively stable low levels. Hence, *SVALKA* would serve as a regulatory hub for *CBF* expression in response to low temperature: through two distinct molecular mechanisms it differentially controls the induction levels of *CBF1* and represses the expression of *CBF3* after its peak of induction, respectively. The biological relevance of this dual functionality of *SVALKA* is evidenced by the fact that both mechanisms are essential to ensure the correct development of the cold acclimation process. Our findings unveil an epigenetic regulation of *CBF* expression in response to low temperature that is mediated by a *SVALKA*-PRC2 module, constitutes a particular layer of *CBF* regulation, and is required for an adequate cold acclimation. Identifying the molecular mechanisms that shape the transient expression patterns of *CBF* genes is a remarkable goal for future studies that should provide insights on how plants respond and adapt to adverse environments.

Materials and methods

Plant materials and growth conditions

Arabidopsis (*A. thaliana*) Col-0 ecotype, which was used in all experiments as the WT plant, and mutant lines *SVK OE* (SALK_007722) and *svk-1* (GK-145A05) were obtained from the Nottingham Arabidopsis Stock Centre. The *clf28swn7* double mutant and the *clf50clf16* double mutant expressing the *35S:GFP:CLF* fusion were kindly provided by José A. Jarillo and Manuel Piñeiro (Del Olmo et al. 2016). The WT plants expressing the *35S:MYC:CLF* fusion (WT *MYC:CLF*) were obtained from Marja C. P. Timmermans (Lodha et al. 2013). *SVK OE* and *svk-1* mutants containing the *35S:MYC:CLF* fusion (*SVK OE MYC:CLF*) were generated by crossing the mutants with WT *MYC:CLF* plants. The binary vector *pMDC45* was introduced in the WT plants via *Agrobacterium tumefaciens* (GV3101 strain) (Clough and Bent 1998), to generate the *35S:GFP* plants. Arabidopsis seeds were surface-sterilized and grown under standard conditions [20 °C under long-day photoperiod (16 h of cool-white, fluorescent light, photon flux of 90 μmol m^{-2} s^{-1})] in pots containing a mixture of organic substrate and vermiculite (3/1, v/v) or in Petri dishes containing Murashige and Skoog medium supplemented with 1% (w/v) sucrose (germination media, GM) and solidified with 0.9% (w/v) plant agar.

Plant treatments and tolerance assays

Low temperature treatments for gene expression analyses and immunoprecipitation experiments were performed by transferring 2-wk-old plants growing in Petri dishes to a growth chamber set at 4 °C for different times under a long-day photoperiod (16 h of cool-white, fluorescent light, photon flux of 40 μmol m^{-2} s^{-1}). In all cases, tissue samples were frozen in liquid nitrogen after treatment and stored at −80 °C until use. Freezing tolerance assays were carried out as previously described (Catala et al. 2011). All data reported about tolerances are expressed as standard deviations of the means of at least three independent experiments with 40 plants each.

Gene expression analysis

For gene expression analyses, total RNA was extracted using TRIzol (Thermo Fisher) according to the manufacturer's protocol. RNA samples were treated with DNase I (Roche) and quantified with a Nanodrop spectrophotometer (Thermo Fisher Scientific). Complementary DNA (cDNA) was synthesized from each sample with the iScript cDNA Synthesis Kit (Bio-Rad) following the manufacturer's instructions, and was used as a template for RT-qPCR assays with the SsoFast EvaGreen Supermix (Bio-Rad) in an iQ2 thermal cycler machine (Bio-Rad) with the primers listed in Supplementary Table S1. In all cases, the relative expression values were calculated using the *AT4G26410* gene as a reference (Czechowski et al. 2005), and the ΔΔCT method to determine fold changes (Livak and Schmittgen 2001). The identification of *SVALKA* splicing isoforms was carried out by RT-qPCR, using cDNA from 2-wk-old WT plants grown in Petri dishes exposed to 4 °C for eight additional hours and primers displayed in Supplementary Table S1. Genomic DNA from the same plants was isolated as described (Cenis 1992) and amplified by PCR with the same primers as a

size reference. All reactions were carried out in triplicate using three independent RNA samples.

Nuclear RNA immunoprecipitation

Nuclear RIP was performed as described (Au et al. 2017) with some modifications. Two-wk-old *35S:GFP:CLF* and *35S:GFP* plants grown in Petri dishes under standard conditions were exposed to 20 °C or 4 °C for eight additional hours, cross-linked with 1% (v/v) formaldehyde and quenched with 0.125 M glycine. After grinding, 10 g of plant material were suspended in 40 mL Honda buffer (Supplementary Table S2). Nuclei were isolated by centrifugation (3,000 × *g*), and the resulting pellet washed four times with Nuclei Wash buffer (Supplementary Table S2), resuspended in Lysis buffer (Supplementary Table S2), and disrupted by sonication in a Sonifier 150D (Branson). One-third of each nuclei lysate was collected as input sample, and the remaining lysate diluted 10 times with Dilution buffer (Supplementary Table S2) and precleared with Pierce Control Agarose Resin (Thermo Scientific). Immunoprecipitation was performed by incubating the precleared nuclei lysate with 20 µL of GFP-Trap beads (Chromotek) o/n at 4 °C. After the incubation, GFP-beads were washed three times with Beads Wash buffer (Supplementary Table S2). RNA-protein complexes were eluted by incubation with 500 µL Elution buffer (Supplementary Table S2) during 1 h at 37 °C in the presence of 20 µg Proteinase K. Immunoprecipitated RNA was extracted using TRIzol (Thermo Fisher) according to the manufacturer's protocol. cDNA synthesis was performed using iScript Advanced cDNA Synthesis kit (Bio-Rad), and quantification of the *SVALKA* β RNA in the samples was performed by RT-qPCR as described above. In all cases, *SVALKA* β RNA levels obtained from the immunoprecipitated samples were relativized to the levels of *SVALKA* β RNA from the corresponding input samples. The primers used to amplify *SVALKA* β RNA are listed in Supplementary Table S1. All data reported are expressed as standard deviations of the means of at least three independent experiments.

Chromatin immunoprecipitation

ChIP experiments were carried out using 2-wk-old plants basically as previously described (Desvoyes et al. 2018). Ten grams of WT *35S:MYC:CLF*, *svk-1 35S:MYC:CLF* and *SVK OE 35S:MYC:CLF* plants, or 2 g of WT, *svk-1* and *SVK OE* plants, were cross-linked with 1% (v/v) formaldehyde by vacuum infiltration and quenched with 0.125 M glycine. After grinding, nuclei were isolated in Extraction buffer (Supplementary Table S2). Nuclei were pelleted by centrifugation (3,000 × *g*), resuspended in Lysis buffer (Supplementary Table S2), and disrupted by sonication in a Sonifier 150D (Branson) to obtain genomic fragments of 100 to 500 bp. One-tenth of each nuclei lysate was collected as input sample, and the remaining lysate was diluted 10 times using Dilution buffer (Supplementary Table S2). DNA/protein complexes were immunoprecipitated with anti-c-MYC monoclonal antibody

(sc-40; Santa Cruz Biotechnology) or anti-H3K27me3 monoclonal antibody (ab6002, Abcam), using magnetic beads (Dynabeads Protein G, Invitrogen). De-cross-linking and DNA purification was carried out using Chelex 100 Resin (Bio Rad). Quantification of chromatin in each sample was determined by qPCR using the primers listed in Supplementary Table S1. In all cases, chromatin levels obtained in the immunoprecipitated samples were relativized to the levels of chromatin in the corresponding input samples. All data reported are expressed as standard deviations of the means of at least three independent experiments.

Accession numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers indicated in Supplementary Table S3.

Author contributions

D.G., R.C., and J.S. conceived and designed the experiments; D.G., J.B., E.T., R.C., and M.F.R. performed the experiments; D.G., R.C., and J.S. analyzed the data; R.C. and J.S. wrote the paper.

Supplementary data

The following materials are available in the online version of this article.

Supplementary Figure S1. The induction of *CBFs* strongly declines after 24 h of cold exposure.

Supplementary Figure S2. The β isoform is the *SVALKA* transcript analyzed in this study.

Supplementary Table S1. Specific primers used in this study.

Supplementary Table S2. Buffers used in this study.

Supplementary Table S3. Full names of the genes mentioned in this study.

Conflict of interest statement: All authors declare that they have no conflicts of interest.

Data availability

The data that support the findings of this study are available from the corresponding authors upon request. All primers used in this work are described in **Supplementary Table S1**. Sequence data from the genes mentioned in this study can be found in the GenBank/EMBL data libraries under the accession numbers listed in **Supplementary Table S3**. The full names of these genes are also included in **Supplementary Table S3**.

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