1   *SVALKA*-POLYCOMB REPRESSIVE COMPLEX2 Module Controls *C-REPEAT*

2   *BINDING FACTOR3* Induction during Cold Acclimation

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38   **Running head:** *SVALKA*-PRC2 module fine-tunes cold acclimation

39

40   **Abstract**

41   C-REPEAT BINDING FACTORS (CBFs) are highly conserved plant transcription factors

42   that promote cold tolerance. In Arabidopsis (*Arabidopsis thaliana*), three CBFs (CBF1-

43   3) play a critical role in cold acclimation, and the expression of their corresponding genes

44   is rapidly and transiently induced during this adaptive response. Cold induction of *CBFs*

45   has been extensively studied and shown to be tightly controlled, yet the molecular

46   mechanisms that restrict the expression of each *CBF* after their induction during cold

47   acclimation are poorly understood. Here, we present genetic and molecular evidence

48   that the decline in the induction of *CBF3* during cold acclimation is epigenetically

49   regulated through the Polycomb Repressive Complex (PRC) 2. We show that this

50   complex promotes the deposition of the repressive mark H3K27me3 at the coding region

51   of *CBF3*, silencing its expression. Our results indicate that the cold-inducible long

52   noncoding RNA *SVALKA* is essential for this regulation by recruiting PRC2 to *CBF3*.

53   These findings unveil a *SVALKA*-PRC2 regulatory module that ensures the precise

54   timing of *CBF3* induction during cold acclimation and the correct development of this

55   adaptive response.

56 **Introduction**

57 Low temperatures adversely affect the growth and development of plants, and condition

58 their geographic distribution and productivity. To cope with low temperatures and acquire

59 freezing tolerance, plants from temperate regions have evolved a sophisticated adaptive

60 response, termed cold acclimation, whereby they increase their freezing tolerance after

61 being exposed to low non-freezing temperatures (Thomashow, 1999). In Arabidopsis

62 (*Arabidopsis thaliana*), crucial to this response are the *C-REPEAT BINDING FACTORS*

63 (*CBFs*), a small family of three tandemly clustered cold-inducible genes (*CBF1-3*)

64 encoding transcription factors that activate the expression of different cold-regulated

65 (*COR*) genes, known as the C-REPEAT BINDING FACTOR (CBF) regulon, to boost

66 freezing tolerance (Gilmour et al., 1998; Jaglo-Ottosen et al., 1998; Liu et al., 1998;

67 Medina et al., 1999; Novillo et al., 2007). The expression of *CBFs* during cold acclimation

68 is characterized by a transient induction with a peak within 3-6 hours of cold exposure

69 and a subsequent rapid decline, after 8-12 hours of exposition, to attain a relatively stable

70 level (Gilmour et al., 1998; Liu et al., 1998; Medina et al., 1999). Despite extensive

71 studies on the regulation of *CBF* cold induction, the molecular mechanisms underlying

72 the posterior decline of expression remain unclear. Here, we show that the Arabidopsis

73 cold-inducible long noncoding RNA *SVALKA*, which is transcribed on the antisense

74 strand between *CBF3* and *CBF1* (Kindgren et al., 2018), recruits the Polycomb

75 Repressive Complex (PRC) 2 to the *CBF3* gene for deposition of the H3K27me3

76 repressive mark, thus restraining its expression after its induction peak and ensuring the

77 correct development of the cold acclimation process. Our study reveals that the

78 expression of *CBFs* during cold acclimation is epigenetically regulated through a

79 *SVALKA*-PRC2 module and this regulation is essential for this adaptive response.

80

81 **RESULTS AND DISCUSSION**

82 Under control conditions, CURLY LEAF (CLF), an essential subunit of the Arabidopsis

83 PRC2 complex, has been associated to the genomic region harboring the *CBFs* (Xiao et

84 al., 2017), and, consistently, the PRC2-dependent repressive mark H3K27me3 is

85 substantially deposited at that region (Sequeira-Mendes et al., 2014). We then decided

86 to explore the possibility that the PRC2 might be involved in repressing the expression

87 of *CBFs* after their induction during cold acclimation. To test this possibility, we assessed

88 the expression of *CBFs* in Arabidopsis *clf28 swn7* mutants exposed 3 or 24 hours to 4

89 °C, times at which they are highly induced or their induction has already drastically

90 decreased in Col-0 (WT) plants, respectively (Supplemental Figure S1). The *clf28 swn7*

91 mutants have a compromised PRC2 activity due to null mutations in *CLF* and *SWN*

92 genes, which encode two of its core subunits, giving rise to small seedlings after

3

93   germination that degenerate to a callus-embryo-like structure (Farrona et al., 2011).

94   Reverse transcription quantitative PCR (RT-qPCR) assays with specific primers for each

95   *CBF* (Figure 1A; Supplemental Table S1) revealed that *CBF1* and *CBF3* transcripts

96   accumulated at higher levels in mutant than in WT plants in response to low temperature,

97   this accumulation being much more significant after 24 hours of cold exposure (Figure

98   1B). The transcript levels of *CBF2*, however, were not affected in the mutants (Figure

99   1B). These data indicated that PRC2 suppresses the expression of *CBF1* and *CBF3*,

100 mainly in the late phase of their induction by low temperature. The induction of *CBF2*

101 does not seem to be silenced by PRC2. Despite their redundant function in cold

102 acclimation, the expression of *CBF* genes is positively and negatively governed through

103 a plethora of common and specific regulators (Ding et al., 2020). Therefore, it is not

104 surprising that PRC2 only silences *CBF1* and *CBF3*. Understanding how *CBF2* gene

105 expression is repressed once it is induced under low temperature conditions requires

106 additional experiments and will be the subject of future studies.

107

108 From the results described above, we anticipated that, after 24 hours of cold exposure,

109 CLF would be substantially associated to *CBF1* and *CBF3* chromatin. This assumption

110 was examined by determining the levels of CLF associated to *CBFs* by means of

111 chromatin immunoprecipitation (ChIP) assays, using an anti-MYC antibody, chromatin

112 isolated from WT transgenic plants expressing a translational *35S:MYC:CLF* fusion

113 (Lodha et al., 2013) grown under control conditions or exposed 24 hours to 4 °C, and

114 specific primers corresponding to different regions of the *CBFs* (Figure 1A; Supplemental

115 Table S1). Results showed that CLF was significantly associated to the *CBF3* coding-

116 region chromatin after 24 hours of cold exposure. The levels of CLF associated to the

117 chromatin upstream of the *CBF3* coding region also resulted to be significantly high after

118 24 hours at 4 °C, although to a lower extent than those detected in the chromatin of the

119 coding region. We did not find a significant association of CLF to the chromatin

120 downstream of the *CBF3* coding region (Figure 1C). Intriguingly, the levels of CLF

121 associated with *CBF1* chromatin were not significant in any of the regions analyzed

122 neither in plants grown under control conditions nor exposed to low temperature. (Figure

123 1C). Consistent with the data described above (Figure 1B), the CLF levels associated

124 with *CBF2* chromatin were not significant either (Figure 1C). Then, we carried out ChIP

125 assays with an anti-H3K27me3 antibody, chromatin from WT plants grown under control

126 conditions or subjected to 4 °C for 24 hours, and the *CBF*-related primers used in the

127 previous experiment (Figure 1C; Supplemental Table S1). Under control conditions,

128 coherently with the low expression of *CBF3*, the levels of the repressive histone mark

129 H3K27me3 were highly significant in its chromatin corresponding to both promoter and

4

130   coding regions (Figure 1D). Although these regions showed very low levels of associated
131   CLF (Figure 1C), this is not surprising considering that around three quarters of the
132   H3K27me3-marked genes have been proposed to not display CLF associated with their
133   chromatin (Shu et al., 2019). These marks would have been deposited by a PRC2
134   containing a different catalytic subunit than CLF, such as SWN7 or MEA, or in a previous
135   developmental stage (Costa and Dean, 2019). Interestingly, after low temperature
136   exposition, the levels of the H3K27me3 histone mark were significantly higher only in the
137   *CBF3* coding-region chromatin (Figure 1D). Altogether, these results strongly suggested
138   that the PRC2 complex represses the expression of *CBF3* during cold acclimation, once
139   attained its induction peak, through the deposition of the H3K27me3 mark at its coding
140   region. The PRC2 complex would also suppress the expression of *CBF1* during cold
141   acclimation but independently of CLF, through another component of the PRC2 like
142   SWN, or through an indirect pathway.

143

144   Now, the arising question was how the PRC2 complex is associated to the chromatin of
145   *CBF3* during cold acclimation. Long noncoding RNAs (lncRNAs) have recently emerged
146   as potent regulators of gene expression and are recognized as important participants in
147   PRC2 function (Margueron and Reinberg, 2011). In Arabidopsis, *COLDAIR*,
148   *COLDWRAP* and *AG-lncRNA4* lncRNAs have been described to physically interact with
149   the PRC2 subunit CLF, contributing to its localization to the corresponding target genes
150   and their subsequent silencing through H3K27me3 deposition (Swiezewski et al., 2009;
151   Kim and Sung, 2017; Wu et al., 2018). A common feature of all these lncRNAs is that
152   they are transcribed from the genomic locus of their target genes (Swiezewski et al.,
153   2009; Kim and Sung, 2017; Wu et al., 2018). Interestingly, as mentioned above, the
154   Arabidopsis genome encodes a cold-induced lncRNA, *SVALKA*, that is transcribed on
155   the antisense strand between *CBF3* and *CBF1*. The transcription takes place from
156   different transcription starting sites, originating short (α) and long (β) isoforms (Kindgren
157   et al., 2018) (Supplemental Figure S2A). Under control conditions, extensive
158   transcription of the *SVALKA* β isoform throughout the *CBF1* gene body generates a
159   natural antisense transcript that negatively regulates *CBF1* transcription (Zacharaki et
160   al., 2023). In response to low temperature, the transcription of *SVALKA* isoforms is
161   induced, reaching a stable maximum of induction after 8-12 hours of treatment (Kindgren
162   et al., 2018). It has been reported that a transcriptional read-through of the *SVALKA* β
163   isoform limits the cold induction of *CBF1* by means of a head-to-head RNA polymerase
164   II (RNAPII) collision mechanism at the 3'-end of this gene, negatively regulating cold
165   acclimation (Kindgren et al., 2018). A function for the *SVALKA* isoforms, however, has
166   not yet been described. Thus, we hypothesized that they could mediate the decline of

167 *CBF3* expression that takes place after its rapid induction during cold acclimation by
168 recruiting PRC2 to *CBF3*. This possibility was assessed by studying the expression
169 levels of *CBF3* in a *SVALKA* T-DNA null mutant (*svk-1*) (Kindgren et al., 2018) exposed
170 to 4 °C for different times. We did not find significant differences in *CBF3* transcript levels
171 between mutant and WT plants after 3 or 8 hours of cold exposure. However, *CBF3*
172 transcripts were significantly higher in *svk-1* than in WT plants after being exposed 24 or
173 72 hours to 4 °C (Figure 2A). Very similar results were obtained when analyzing the
174 *SVALKA* T-DNA mutant *SVK OE*, which overexpresses a partial and aberrant *SVALKA*
175 transcript (Kindgren et al., 2018) (Figure 2A). Our results, therefore, unveiled a role for
176 *SVALKA* consisting in promoting the decline of *CBF3* expression that takes place after
177 its induction during cold acclimation. It is worth mentioning that, as displayed in Figure
178 1B, *clf28 swn7* mutations lead to increased expression of *CBF3* at both 3 and 24 hours
179 after cold treatment, but disruption of *SVALKA* only affects *CBF3* expression after 24
180 hours of exposure at 4 °C (Figure 2A). Considering that *SVALKA* is not expressed after
181 3 hours of cold treatment (Kindgren et al., 2018), we can conclude that the effect of PRC2
182 on *CBF3* expression in the early stage of cold acclimation is not mediated by *SVALKA*.

184 As shown above (Figure 2A), *svk-1* and *SVK OE* mutants had in common a delay in the
185 decline of *CBF3* induction during cold acclimation compared to WT plants. Interestingly,
186 however, they have been described to diverge in the induction levels of *CBF1*. Indeed,
187 while the cold induction of *CBF1* is increased in *svk-1*, it is significantly reduced in *SVK*
188 *OE* (Kindgren et al., 2018). The *SVK OE* mutant, therefore, constitutes a very good
189 material to evaluate the physiological significance of the role unveiled for the *SVALKA*
190 lncRNA. First, we determine the mRNA levels corresponding to some CBF-activated
191 *COR* genes such as *KIN1*, *XERO2*, *GOLS3* and *COR15B* in *SVK OE* plants subjected
192 to low temperature for 24 and 72 hours. *svk-1* mutants were also included in the analysis
193 as a reference. Consistent with the high levels of *CBF3* transcripts present in *SVK OE*
194 and *svk-1* plants after a long-term cold treatment (Figure 2A), the expression of *COR*
195 genes was significantly higher in both mutants than in WT plants exposed 72 hours to 4
196 °C (Figure 2B). Then, we studied the effect of the late up-regulation of *CBF3* and *COR*
197 gene expression on the ability of *SVK OE* plants to tolerate freezing and cold acclimate.
198 In this study, *svk-1* mutants were again included for reference. Two-week-old
199 nonacclimated and cold acclimated (7 additional days at 4 °C) mutant plants were
200 subjected to decreasing freezing temperatures and, after 2 weeks of recovery under
201 control conditions, scored for survival. Nonacclimated mutants presented a similar
202 capacity to tolerate freezing as the WT, the LT_{50} (temperature that causes 50% lethality)
203 values being in both cases around -6.8 °C (Figure 2C). By contrast, cold acclimated

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204 mutants exhibited a significantly higher freezing tolerance than cold acclimated WT

205 plants, the LT_{50} values in this case being -10.8 °C for both *SVK OE* and *svk-1* mutants

206 and -10.0 °C for WT plants (Figure 2D). As already mentioned, the expression of *CBF1*

207 is only slightly induced by low temperature in the *SVK OE* mutant (Kindgren et al., 2018).

208 Therefore, our findings clearly indicated that *SVALKA* also contributes to the correct

209 development of cold acclimation by mediating the decline of *CBF3* induction during this

210 adaptive response.

211

212 Next, we studied if *SVALKA* could contribute to suppress the induction of *CBF3* during

213 cold acclimation by targeting PRC2 to this gene. First, the possible interaction of

214 *SVALKA* with CLF was analyzed by means of RNA immunoprecipitation (RIP) assays.

215 We used null *clf* double mutant plants *clf50 clf16* expressing a *35S:GFP:CLF* fusion

216 (Schubert et al., 2006) grown under control conditions or exposed 8 hours to 4 °C, a time

217 at which the expression of *SVALKA* is highly induced (Kindgren et al., 2018), and an anti-

218 GFP antibody. Plants WT expressing a *35S:GFP* fusion were used as a control for

219 specificity. The presence of *SVALKA* transcripts in the immunoprecipitates was

220 determined and quantified by RT-qPCR with specific primers (Figure S2A; Supplemental

221 Table S1) that, under our experimental conditions, solely amplify the spliced β isoform

222 (Figure S2B). We only detected a significant enrichment of this isoform in the

223 immunoprecipitated fraction corresponding to samples from plants expressing the

224 *35S:GFP:CLF* fusion exposed to 4 °C (Figure 3A), indicating that, indeed, *SVALKA*

225 physically interacts with CLF in response to low temperature. Then, we assessed

226 whether the interaction of *SVALKA* with CLF was required for targeting this protein, and

227 therefore PRC2, to the *CBF3* coding region. Chromatin immunoprecipitation experiments

228 were performed with an anti-MYC antibody, chromatin isolated from *svk-1*, *SVK OE* and

229 WT plants expressing the *35S:MYC:CLF* fusion grown under control conditions or

230 exposed 24 hours to 4 °C, and the specific *CBF3* primers corresponding to its coding

231 region (Figure 1A, Supplemental Table S1). The levels of MYC:CLF associated to the

232 chromatin of *CBF1* coding-region in WT, *svk-1* and *SVK OE* plants under control and

233 cold conditions were also quantified as internal controls in the experiments. The absence

234 of a functional *SVALKA* in *svk-1* and *SVK OE* plants did not affect the levels of CLF

235 associated with the *CBF3* chromatin in plants grown at 20 °C (Figure 3B). After 24 hours

236 of cold exposure, however, the lack of a functional *SVALKA* led to significantly lower

237 levels of associated CLF than in WT plants, similar to those observed under control

238 conditions (Figure 3B). As expected, the levels of CLF associated with the *CBF1*

239 chromatin in *svk-1* and *SVK OE* mutants were, in all cases, as in WT plants, which, in

240 turn, were similar to those associated to *CBF3* chromatin in WT plants grown under

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control conditions (Figure 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 *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 24 hours to 4 °C. The levels of H3K27me3 at the chromatin of *CBF1* were also quantified in these plants as an internal control. Chromatin immunoprecipitation assays using an anti-H3K27me3 antibody and specific primers for *CBF1* and *CBF3* coding regions (Figure 1A, Supplemental 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 WT plants (Figure 3C). Consistent with the results described above (Figure 3B), the levels of H3K27me3 at *CBF1* were similar in all genotypes (Figure 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 Figure 3D. In response to low temperature, the expression of *CBFs* is rapidly induced, after 15-30 minutes, reaching the highest levels at 3-4 hours 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 8-12 hours 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 hours 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

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278   unveil an epigenetic regulation of *CBF* expression in response to low temperature that is
279   mediated by a *SVALKA*-PRC2 module, constitutes a particular layer of *CBF* regulation,
280   and is required for an adequate cold acclimation. Identifying the molecular mechanisms
281   that shape the transient expression patterns of *CBF* genes is a remarkable goal for future
282   studies that should provide insights on how plants respond and adapt to adverse
283   environments.

284

MATERIALS AND METHODS
Plant materials and growth conditions
287   Arabidopsis (*Arabidopsis thaliana*) Col-0 ecotype, which was used in all experiments as
288   WT plant, and mutant lines *SVK OE* (SALK_007722) and *svk-1* (GK-145A05) were
289   obtained from the Nottingham Arabidopsis Stock Centre. The *clf28swn7* double mutant
290   and the *clf50clf16* double mutant expressing the *35S:GFP:CLF* fusion were kindly
291   provided by José A. Jarillo and Manuel Piñeiro (Del Olmo et al., 2016). WT plants
292   expressing the *35S:MYC:CLF* fusion (WT *MYC:CLF*) were obtained from Marja C. P.
293   Timmermans (Lodha et al., 2013). *SVK OE* and *svk-1* mutants containing the
294   *35S:MYC:CLF* fusion (*SVK OE MYC:CLF*) were generated by crossing the mutants with
295   WT *MYC:CLF* plants. The binary vector *pMDC45* was introduced in WT plants via
296   *Agrobacterium tumefaciens* (GV3101 strain) (Clough and Bent, 1998), to generate the
297   *35S:GFP* plants. Arabidopsis seeds were surface-sterilized and grown under standard
298   conditions [20°C under long-day photoperiod (16 hours of cool-white, fluorescent light,
299   photon flux of 90 µmol m^{-2} s^{-1})] in pots containing a mixture of organic substrate and
300   vermiculite (3/1, v/v) or in petri dishes containing Murashige and Skoog medium
301   supplemented with 1% (w/v) sucrose [germination media (GM)] and solidified with 0.9%
302   (w/v) plant agar.

303

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

313

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 Supplemental 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-week-old WT plants grown in petri dishes exposed 8 additional hours to 4 °C and primers displayed in Supplemental 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 RNA immunoprecipitation was performed as described (Au et al., 2017) with some modifications. Two-week-old *35S:GFP:CLF* and *35S:GFP* plants grown in petri dishes under standard conditions were exposed for 8 additional hours to 20 °C or 4 °C, cross-linked with 1% (v/v) formaldehyde and quenched with 0.125 M glycine. After grinding, 10 gr of plant material were suspended in 40 ml Honda buffer (Supplemental Table S2). Nuclei were isolated by centrifugation (3,000g), and the resulting pellet washed four times with Nuclei Wash buffer (Supplemental Table S2), resuspended in Lysis buffer (Supplemental 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 ten times with Dilution buffer (Supplemental Table S2) and pre-cleared with Pierce™ Control Agarose Resin (Thermo Scientific). Immunoprecipitation was performed by incubating the pre-cleared nuclei lysate with 20 µl of GFP-Trap beads (Chromotek) o/n at 4 °C. After the incubation, GFP-beads were washed 3 times with Beads Wash buffer (Supplemental Table S2). RNA-protein complexes were eluted by incubation with 500 µl Elution buffer (Supplemental Table S2) during 1 hour 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

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351   all cases, *SVALKA* β RNA levels obtained from the immunoprecipitated samples were
352   relativized to the levels of *SVALKA* β RNA from the corresponding input samples. The
353   primers used to amplify *SVALKA* β RNA are listed in Supplemental Table S1. All data
354   reported are expressed as standard deviations of the means of at least three
355   independent experiments.
356

357   **Chromatin Immunoprecipitation**
358   Chromatin immunoprecipitation (ChIP) experiments were carried out using 2-week-old
359   plants basically as previously described (Desvoyes et al., 2018). Ten gr of WT
360   *35S:MYC:CLF*, *svk-1 35S:MYC:CLF* and *SVK OE 35S:MYC:CLF* plants, or 2 gr of WT,
361   *svk-1* and *SVK OE* plants, were cross-linked with 1% (v/v) formaldehyde by vacuum
362   infiltration and quenched with 0.125 M glycine. After grinding, nuclei were isolated in
363   Extraction buffer (Supplemental Table S2). Nuclei were pelleted by centrifugation
364   (3,000g), resuspended in Lysis buffer (Supplemental Table S2), and disrupted by
365   sonication in a Sonifier® 150D (Branson) to obtain genomic fragments of 100 to 500 bp.
366   One tenth of each nuclei lysate was collected as input sample, and the remaining lysate
367   was diluted ten times using Dilution buffer (Supplemental Table S2). DNA/protein
368   complexes were immunoprecipitated with anti-c-MYC monoclonal antibody (sc-40;
369   Santa Cruz Biotechnology) or anti-H3K27me3 monoclonal antibody (ab6002, Abcam),
370   using magnetic beads (Dynabeads Protein G, Invitrogen). De-cross-linking and DNA
371   purification was carried out using Chelex 100 Resin (Bio Rad). Quantification of
372   chromatin in each sample was determined by qPCR using the primers listed in
373   Supplemental Table S1. In all cases, chromatin levels obtained in the
374   immunoprecipitated samples were relativized to the levels of chromatin in the
375   corresponding input samples. All data reported are expressed as standard deviations of
376   the means of at least three independent experiments.
377

378   **Accession Numbers**
379   Sequence data from this article can be found in the GenBank/EMBL data libraries
380   under accession numbers indicated in Supplemental Table S3.
381

382   **Supplemental Data**
383   Supplemental Figure S1. The induction of *CBFs* strongly declines after 24 hours of cold
384   exposure.
385   Supplemental Figure S2. The β isoform is the *SVALKA* transcript analyzed in this
386   study.
387   Supplemental Table S1. Specific primers used in this study.

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388 Supplemental Table S2. Buffers used in this study.

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

390

391 **Data availability**

392 The data that support the findings of this study are available from the corresponding

393 authors upon request. All primers used in this work are described in Supplemental Table

394 S1. Sequence data from the genes mentioned in this study can be found in the

395 GenBank/EMBL data libraries under the accession numbers listed in Supplemental

396 Table S3. The full names of these genes are also included in Supplemental Table S3.

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409 **Author contributions**

410 D.G., R.C. and J.S. conceived and designed the experiments. D.G., J.B., E.T. R.C. and

411 M.F.R. performed the experiments. D.G., R.C. and J.S. analyzed the data. R.C. and J.S.

412 wrote the paper.

413

414 **Conflict of interest**

415 All authors declare that they have no conflicts of interest.

416

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Figure legends

417

Figure 1. The cold induction of *CBF3* is repressed by the PRC2 complex.

418

419   **A**, Schematic representation of the Arabidopsis genomic region containing the *CBF*

420   genes. Arrowheads indicate the relative positions of primer sets used for expression

421   analyses and ChIP experiments in this work. Each primer set is identified by a number.

422   **B**, Expression analysis of *CBF1*, *CBF3* and *CBF2* in 2-week-old Col-0 (WT) and *clf28*

423   *swn7* plants maintained or exposed 3 or 24 additional hours to 20 or 4 °C, respectively.

424   In each condition (20 or 4 °C), transcript levels, determined by RT-qPCR, are

425   represented as relative to the values of the corresponding WT. Asterisks indicate

426   significant differences (**$P<0.01$, ***$P<0.001$, ****$P<0.0001$) between *clf28 swn7* and WT

427   in each condition, as determined by two-sided *t* test. The numbers in parentheses

428   indicate the primer sets used. **C** and **D**, ChIP experiments showing the levels of MYC-

429   CLF **(C)** and H3K27me3 **(D)** on the chromatin of *CBFs* from 2-week-old WT plants grown

430   24 additional hours at 20 or 4 °C. The relative amounts of DNA in the input and the

431   immunoprecipitated samples were determined by qPCR with the *CBF* primer sets

432   indicated below the bars in three biological replicates per sample. The levels of MYC-

433   CLF and H3K27me3 were determined as the percentage of input DNAs recovered in the

434   immunoprecipitates. Distinct letters indicate significant differences ($P<0.05$) according to

435   one-way ANOVA followed by Tukey's test. In **B**, **C** and **D**, data represent the mean of

436   three independent experiments and error bars show the standard deviation.

437

438   **Figure 2. *SVALKA* regulates the cold acclimation response by mediating the**

439   **decline of *CBF*3 induction.**

440   **A** and **B**, Expression analysis of *CBF3* **(A)** and CBF3 target genes *KIN1*, *XERO2*,

441   *GOLS2* and *COR15B* **(B)** in 2-week-old WT, *svk-1* and *SVK OE* plants exposed to 4 °C

442   for the indicated additional hours (3h, 8h, 24h or 72h). In each case, transcript levels,

443   determined by RT-qPCR, are represented as relative to the values of the WT plants

444   grown under control conditions. Primer set 5 in Figure 1A was used for expression

445   analyses of *CBF3*. **C** and **D**, Freezing tolerance of non-acclimated **(C)** and cold

446   acclimated 7 days at 4 °C **(D)** 2-week-old WT, *svk-1* and *SVK OE* plants exposed to the

447   indicated freezing temperatures for 6 h. Tolerance was estimated as the percentage of

448   plants surviving each specific temperature after one week of recovery under control

449   conditions. Lower panels show the freezing tolerance of representative non-acclimated

450   and cold acclimated plants. Images were digitally extracted for comparison. In all panels,

451   asterisks indicate significant differences (*$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$)

452   between *svk-1* and *SVK OE* mutants and the corresponding WT plants, as determined

13

453 by one-sided *t* test. Data represent the mean of three independent experiments and error

454 bars show the standard deviation.

455

456 **Figure 3. *SVALKA* interacts with CLF targeting PRC2 to *CBF3* for H3K27me3**

457 **deposition.**

458 **A**, RIP assays performed with 2-week-old *clf50 clf16* plants expressing a *35S:GFP:CLF*

459 fusion maintained or exposed 8 additional hours to 20 or 4 °C, respectively, and an anti-

460 GFP antibody. Two-week-old Col-0 (WT) plants expressing a *35S:GFP* fusion were used

461 as interaction specificity controls. The relative amounts of *SVALKA* lncRNA (β isoform)

462 in the input and the immunoprecipitated samples were determined by RT-qPCR, with

463 three biological replicates per sample. The enrichment of *SVALKA* in the samples was

464 determined as the percentage of input RNA recovered in the immunoprecipitates, and is

465 represented relative to the enrichment in the corresponding *35S:GFP* samples. **B** and **C**,

466 ChIP experiments showing the levels of MYC-CLF on the *CBF3* and *CBF1* chromatin of

467 2-week-old WT, *svk-1* and *SVK OE* plants expressing the *MYC:CLF* fusion maintained

468 at 20 °C or exposed 24h to 4 °C **(B)**, and the levels of H3K27me3 on the *CBF3* and *CBF1*

469 chromatin of 2-week-old WT, *svk-1* and *SVK OE* plants exposed 24h to 4 °C **(C)**. The

470 relative amounts of DNA in the input and the immunoprecipitated samples were

471 determined by qPCR, with the *CBF* primer sets indicated in parenthesis (see Figure 1A),

472 in three biological replicates per sample. The levels of MYC-CLF and H3k27me3 were

473 determined as the percentage of input DNA recovered in the immunoprecipitates. **D**,

474 Proposed model for *SVALKA* function in Arabidopsis cold acclimation. In **A** and **C**,

475 asterisks indicate significant differences (**P<0.01, ***P<0.001) between *35S:GFP:CLF*

476 and *35S:GFP*, and *svk-1* or *SVK OE* and WT in *CBF3*, respectively, as determined by

477 one-sided *t* test. In **B**, asterisk indicates significant differences (*P<0.05) between *svk-1*

478 *MYC:CLF* or *SVK OE MYC:CLF* and WT *MYC:CLF* in *CBF3* at 20 °C, as determined by

479 one-way ANOVA followed by Tukey's test. Data represent the mean of three

480 independent experiments and error bars show the standard deviation.

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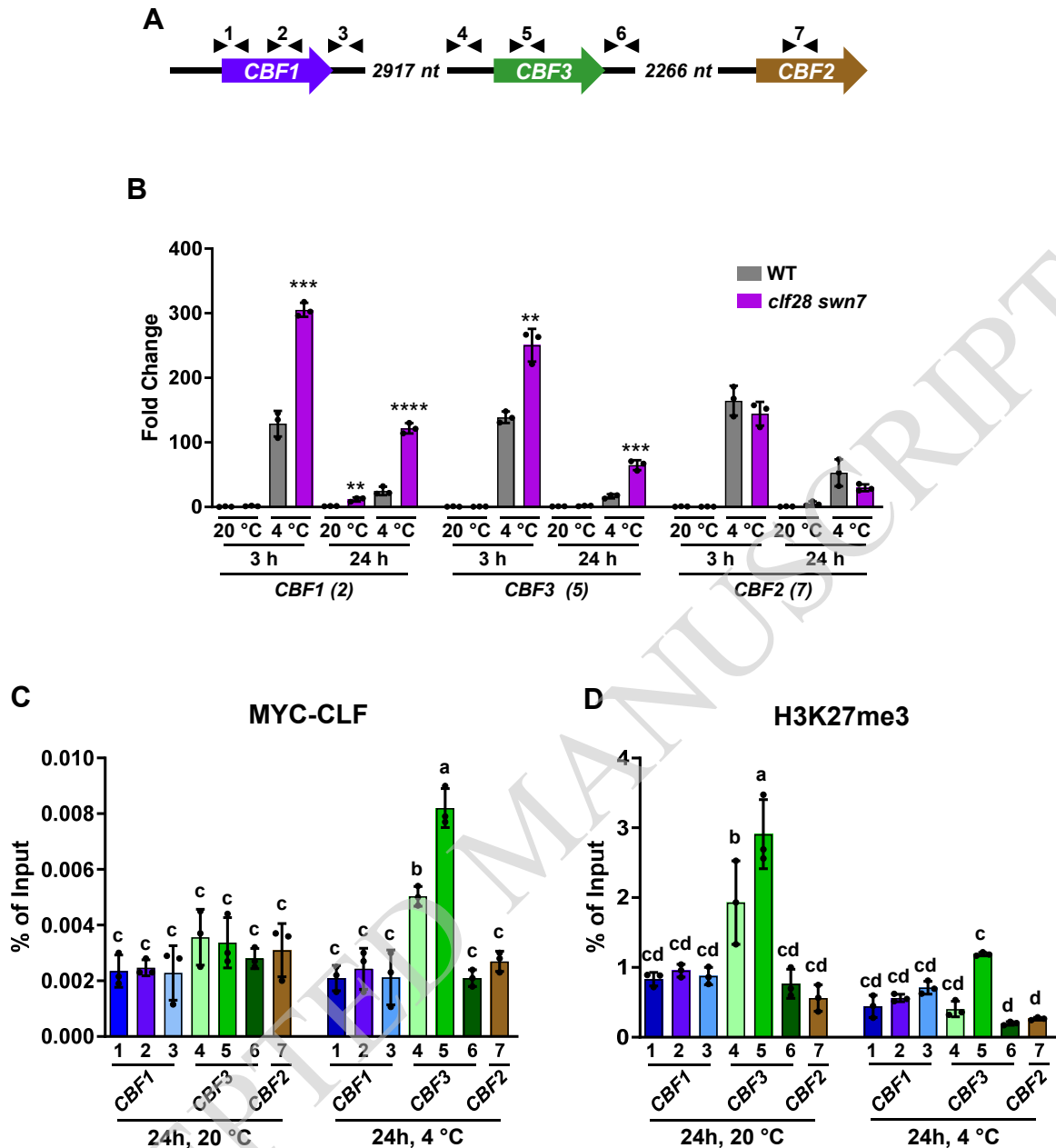568   10.1016/j.xplc.2023.100551

Figure 1

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-week-old Col-0 (WT) and *clf28 swn7* plants maintained or exposed 3 or 24 additional hours to 20 or 4 °C, 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-week-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 RT-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.
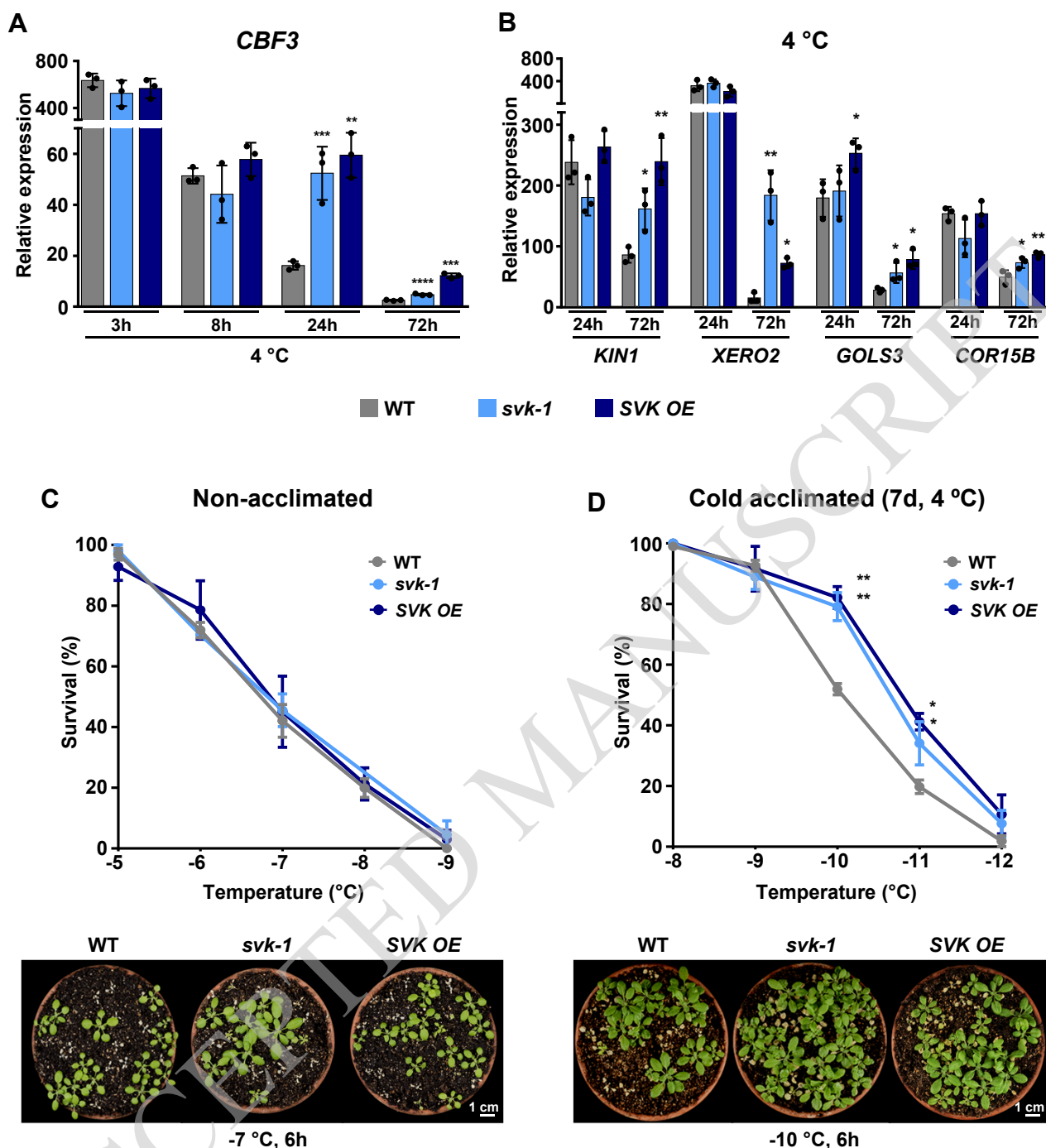
Figure 2

Figure 2. *SVALKA* regulates the cold acclimation response by mediating the decline of *CBF3* induction.
A and **B**, Expression analysis of *CBF3* **(A)** and CBF3 target genes *KIN1*, *XERO2*, *GOLS2* and *COR15B* **(B)** in 2-week-old WT, *svk-1* and *SVK OE* plants exposed to 4 °C for the indicated additional hours (3h, 8h, 24h or 72h). 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 Figure 1A was used for expression analyses of *CBF3*. **C** and **D**, Freezing tolerance of non-acclimated **(C)** and cold acclimated 7 days at 4 °C **(D)** 2-week-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 one week of recovery under control conditions. Lower panels show the freezing tolerance of representative non-acclimated 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.
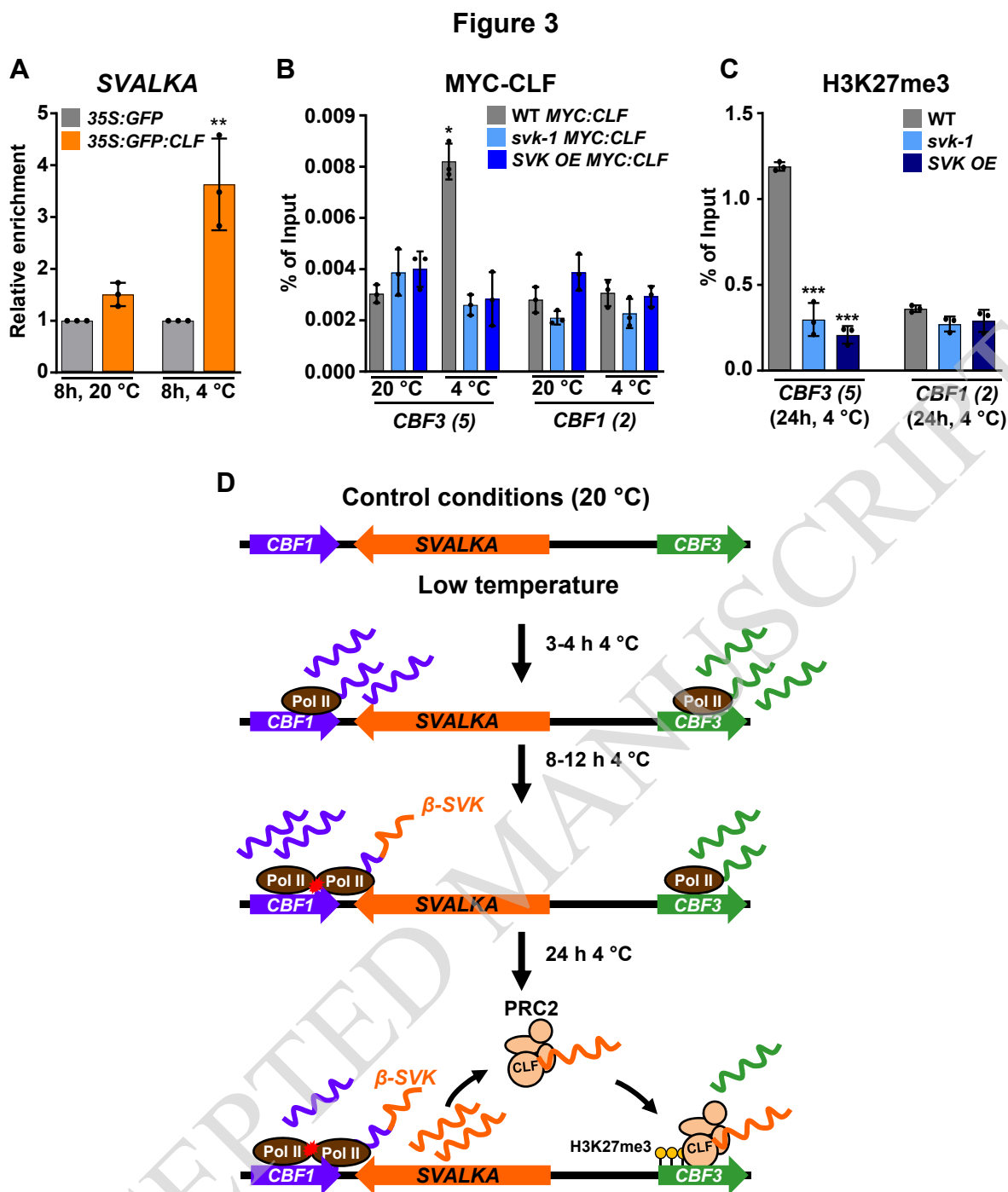
Figure 3

Figure 3. *SVALKA* **interacts with CLF targeting PRC2 to** *CBF3* **for H3K27me3 deposition.**
A, RIP assays performed with 2-week-old *clf50 clf16* plants expressing a *35S:GFP:CLF* fusion maintained or exposed 8 additional hours to 20 or 4 °C, respectively, and an anti-GFP antibody. Two-week-old 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-week-old WT, *svk-1* and *SVK OE* plants expressing the *MYC:CLF* fusion maintained at 20 °C or exposed 24h to 4 °C **(B)**, and the levels of H3K27me3 on the *CBF3* and *CBF1* chromatin of 2-week-old WT, *svk-1* and *SVK OE* plants exposed 24h to 4 °C **(C)**. The relative amounts of DNA in the input and the immunoprecipitated samples were determined by RT-qPCR, with the *CBF* primer sets indicated in parenthesis (see Figure 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.