

# Temperature modulation of CAMTA3 gene induction activity is mediated through the DNA binding domain

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Received 27 January 2022; revised 30 July 2022; accepted 2 August 2022; published online 12 August 2022.

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## SUMMARY

The calmodulin-binding transcription activator (CAMTA) proteins of *Arabidopsis thaliana* play a major role in cold acclimation, contributing to the rapid induction of the *C-REPEAT BINDING FACTOR* (*CBF*) genes and other genes that impart freezing tolerance in plants exposed to cold temperature (4°C). The goal of this study was to better understand how the gene induction activity of CAMTA3 is modulated by temperature. Our results indicate that a severely truncated version of CAMTA3, CAMTA3<sup>334</sup>, which includes the N-terminal CG-1 DNA binding domain and a newly identified transcriptional activation domain (TAD), was able to rapidly induce the expression of *CBF2* and two newly identified target genes, *EXPANSIN-LIKE A1* (*EXPL1*) and *NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 3* (*NCED3*), in response to cold temperature. Additionally, CAMTA3<sup>334</sup> was able to restore freezing tolerance when expressed in a *camta23* double mutant. The ability of CAMTA3 and CAMTA3<sup>334</sup> to induce target genes at cold temperature did not involve increased levels of these proteins or increased binding of these proteins to target gene promoters in cold-treated plants. Rather, domain-swapping experiments indicated that the CAMTA3 CG-1 domain conferred temperature dependence to the ability of the CAMTA3 TAD to induce gene expression. The CG-1 domain also enabled the TAD to induce the expression of target genes at a moderate temperature (22°C) in response to cycloheximide treatment, consistent with the TAD activity not being intrinsically temperature dependent. We propose a working model in which the temperature modulation of CAMTA3 gene induction activity occurs independently from the C-terminal calmodulin-binding domains that previously have been proposed to activate CAMTA3 transcriptional activity in response to cold temperature.

**Keywords:** CAMTA3, *Arabidopsis*, cold acclimation, SA-mediated immunity, transcriptional activation and repression.

## INTRODUCTION

Freezing temperatures limit the geographical distribution of plants in nature and cause significant losses in crop productivity on an annual basis. Plants vary greatly in their maximum level of freezing tolerance (Thomashow, 1999; Zuther et al., 2018): for instance, whereas *Solanum lycopersicum* (tomato) is unable to survive the slightest freeze, *Triticum aestivum* (wheat) can survive freezing at about –20°C. However, the level of freezing tolerance is not a constant property, as plants from temperate regions such as wheat and *Arabidopsis thaliana* (hereafter referred to as *Arabidopsis*) increase in freezing tolerance upon exposure to non-freezing cold temperatures, a process known as cold acclimation (Knight & Knight, 2012; Thomashow, 2010). A fundamental goal of cold acclimation research is

to determine how plants sense and transduce cold signals to activate the gene regulatory networks that increase plant freezing tolerance, and to use this information to develop new approaches to improve the cold stress tolerance of crop plants.

The *Arabidopsis* C-repeat binding factor (CBF) response pathway is a well-studied regulatory pathway with a major role in cold acclimation (Barrero-Gil & Salinas, 2018; Shi et al., 2018). When plants are exposed to non-freezing cold temperatures, three *CBF* genes, *CBF1*, *CBF2* and *CBF3* (also known as *DREB1b*, *DREB1c* and *DREB1a*, respectively), are induced within 15 min (Gilmour et al., 1998; Jaglo-Ottosen et al., 1998; Kasuga et al., 1999; Liu et al., 1998; Stockinger et al., 1997), followed by the induction of about 100 downstream cold-regulated genes

(CORs), termed the CBF regulon (Fowler & Thomashow, 2002; Maruyama et al., 2004; Vogel et al., 2005). The three CBF genes, which are physically linked in tandem array, encode closely related members of the APETALA 2/ethylene-responsive element binding factor (AP2/ERF) family of transcription factors that recognize the C-repeat/dehydration-responsive element (CRT/DRE) cis-element (rCCGAC) present in the promoter regions of target CBF regulon genes (Sakuma et al., 2002). Cold induction of the CBF genes results in expression of the CBF regulon and activation of mechanisms that confer freezing tolerance (Jia et al., 2016; Park et al., 2018; Zhao et al., 2016).

The calmodulin-binding transcription activator (CAMTA) proteins, also known as signal response (SR) proteins, also have an important role in cold acclimation, contributing to the induction of approximately 15% of the genes that are cold induced at 24 h and accounting for about 50% of the increase in freezing tolerance that occurs in response to cold temperature (Kim et al., 2013). Arabidopsis has six CAMTA proteins, four of which – CAMTA1, CAMTA2, CAMTA3 and CAMTA5 – contribute to the rapid cold induction of *CBF1*, *CBF2* and *CBF3* (Doherty et al., 2009; Kidokoro et al., 2017; Kim et al., 2013). The CAMTA proteins are highly conserved among multicellular eukaryotes and have been identified in over 40 plant species, ranging from moss to flowering plants (Rahman et al., 2016). Each CAMTA protein has four functional domains positioned in the same relative order from the N- to C-terminal end: a CG-1 DNA binding domain, an ankyrin (ANK) repeat domain composed of two ANK repeats, an IQ domain composed of two IQ motifs, and a calmodulin-binding (CaMB) domain (Bouché et al., 2002; Finkler et al., 2007). Both the IQ and CaMB domains bind calmodulin in a calcium-dependent manner (Bouché et al., 2002; Choi et al., 2005; Du et al., 2009; Nie et al., 2012). The CG-1 domain recognizes the CG-1 DNA motif vCGC/TGb that is present in the promoters of *CBF1*–*CBF3* and other target genes (Choi et al., 2005; Finkler et al., 2007; Kim et al., 2013). It is not known how the CAMTA proteins induce expression of the CBFs and other target genes in response to cold temperature. However, we have suggested (Doherty et al., 2009) that the increase in cytosolic calcium levels triggered by exposing plants to cold temperature (Knight et al., 1991) might lead to the formation of a Ca<sup>2+</sup>–calmodulin complex that binds to CAMTA3, presumably at the IQ and/or CaMB domains, and enables it to induce the expression of CBF and other target genes.

The CAMTA proteins not only have an important role in cold acclimation, but also in regulating salicylic acid (SA)-mediated plant immunity. In particular, in healthy Arabidopsis plants grown at moderate temperature, CAMTA1, CAMTA2 and CAMTA3 (hereafter CAMTA123) function in an additive manner to repress the biosynthesis of SA and the expression of pathogen defense genes (Du et al., 2009;

Galon et al., 2008; Kidokoro et al., 2017; Kim et al., 2013). The CAMTA-mediated repression of SA biosynthesis results, at least in part, from CAMTA123 repressing the expression of *CBP60g* and *SARD1*, which encode transcription factors that directly induce the expression of *ISOCHORISMIC ACID SYNTHASE 1 (ICS1)*, the key enzyme controlling the primary rate-limiting step in SA biosynthesis (Dempsey et al., 2011; Wildermuth et al., 2001). However, upon exposing plants to cold temperature for more than 1–2 weeks, the ability of the CAMTA proteins to repress *CBP60g* and *SARD1* is suppressed by an unknown mechanism, resulting in the induction of *ICS1*, the biosynthesis of SA and the SA-mediated induction of *PR1* and other defense genes (Kim et al., 2013). Notably, a severely truncated variant of CAMTA3, designated CAMTA3<sup>334</sup>, which comprises the first 334 amino acids of CAMTA3, including the N-terminal DNA binding domain, was found to be highly effective in repressing the expression of SA pathway genes in plants grown at moderate temperature and in plants exposed to cold temperature for as long as 3 weeks (Kim et al., 2017). As CAMTA3<sup>334</sup> does not include C-terminal IQ and CaMB domains, it was concluded that the N-terminal region of CAMTA3 comprises an N-terminal repression module (NRM) that can repress the expression of genes involved in SA biosynthesis independently of Ca<sup>2+</sup>–calmodulin binding to the protein (Kim et al., 2017).

The goal of this current study was to gain a greater understanding of how CAMTA3 rapidly induces the expression of target genes in response to cold temperature (4°C). Our results indicate that the CAMTA3 NRM includes a functional transcriptional activation domain (TAD) and is sufficient to induce target genes in response to cold temperature. The fact that the CAMTA3 NRM does not include the CAMTA3 IQ and CaMB domains indicates that the temperature-dependent activity of the TAD does not require Ca<sup>2+</sup>–calmodulin interaction with these regions of CAMTA3. Rather, our results indicate that the CAMTA3 CG-1 DNA binding region confers temperature dependence upon the TAD. We propose a model in which a suppressor protein interacts with the CG-1 DNA binding region and inhibits TAD activity at a moderate temperature (22°C).

## RESULTS

### CAMTA3 rapidly induces the expression of *EXPL1* and *NCED3* in response to cold temperature

Previous studies established that the rapid cold induction of *CBF1*, *CBF2* and *CBF3* is impaired, but not eliminated, in *camta12*, *camta13* and *camta23* double mutants (Doherty et al., 2009; Kim et al., 2013). Moreover, Kidokoro et al. (2017) found that the CBF genes are significantly induced by cold temperature even in *camta123456* sextuple mutants. These findings are not surprising given that more than 10 transcription factors have been shown to

regulate the expression of the *CBF* genes (Shi et al., 2018). This complexity complicates using the *CBF* genes as a model to determine how CAMTA transcription factors induce genes in response to cold temperature. Therefore, we sought to identify genes that were more dependent on CAMTA transcription factors for cold induction than were the *CBF* genes. Candidate genes were picked among those shown to be induced by cold treatment for 3 h (Kilian et al., 2007) and significantly impaired by cold induction in a *camta123* triple mutant exposed to cold temperature for 24 h (Kim et al., 2013). Among these genes were *EXPANSIN-LIKE A1 (EXPL1)*, *SMALL AUXIN UPREGULATED RNA 79 (SAUR79)* and *NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 3 (NCED3)*. We tested the induction kinetics of these genes in response to cold temperature in wild-type plants (Col-0) and in the *camta123* mutant and found that the profiles for *EXPL1* and *SAUR79* were very similar to that of *CBF2*, but that their induction was impaired to a greater degree in the *camta123* mutant than was *CBF2* (Figure 1a). *NCED3* was also rapidly induced in response to cold temperature, but instead of reaching peak expression at about 3 h, the transcript levels continued to increase up to at least 24 h. However, like *EXPL1* and *SAUR79*, the rapid induction of *NCED3* was considerably impaired in the *camta123* mutant (Figure 1a).

It has been shown that *camta123* plants have extremely high levels of SA through the induction of genes involved in SA biosynthesis (Kim et al., 2013). To determine whether the impaired cold induction of *CBF2*, *EXPL1*, *SAUR79* and *NCED3* in the *camta123* mutant was linked to the high levels of SA in these plants, we determined the transcript levels of these genes in a *camta123 sid2-1* mutant, which has low levels of SA, similar to those found in wild-type plants (Kim et al., 2020); *sid2-1* is a non-functional allele of *ICS1* that encodes the primary rate-limiting step in SA biosynthesis in Arabidopsis (Wilder-muth et al., 2001). The results indicated that the cold induction of *CBF2*, *EXPL1*, *SAUR79* and *NCED3* was greatly impaired in the *camta123 sid2-1* plants indicating that their impaired induction in the *camta123* plants was not linked to the high levels of SA in these plants (Figure 1b).

CAMTA1, CAMTA2 and CAMTA3 act additively to rapidly induce the expression of *CBF1*, *CBF2* and *CBF3* in response to cold temperature (Kim et al., 2013). To determine whether CAMTA1, CAMTA2 and CAMTA3 also functioned additively in the cold induction of *EXPL1*, *SAUR79* and *NCED3*, we tested the expression of these genes in single, double and triple mutants of *camta1*, *camta2*, and *camta3*. We found that the expression level of each gene progressively decreased in the double and triple *camta* mutants, with the order from least to most affected being *camta12*, *camta13*, *camta23* and *camta123*, indicating that all three CAMTA proteins contributed to the cold induction of *EXPL1*, *SAUR79* and *NCED3*, and that CAMTA3

contributed the most, followed by CAMTA2 and CAMTA1 (Figure 1c).

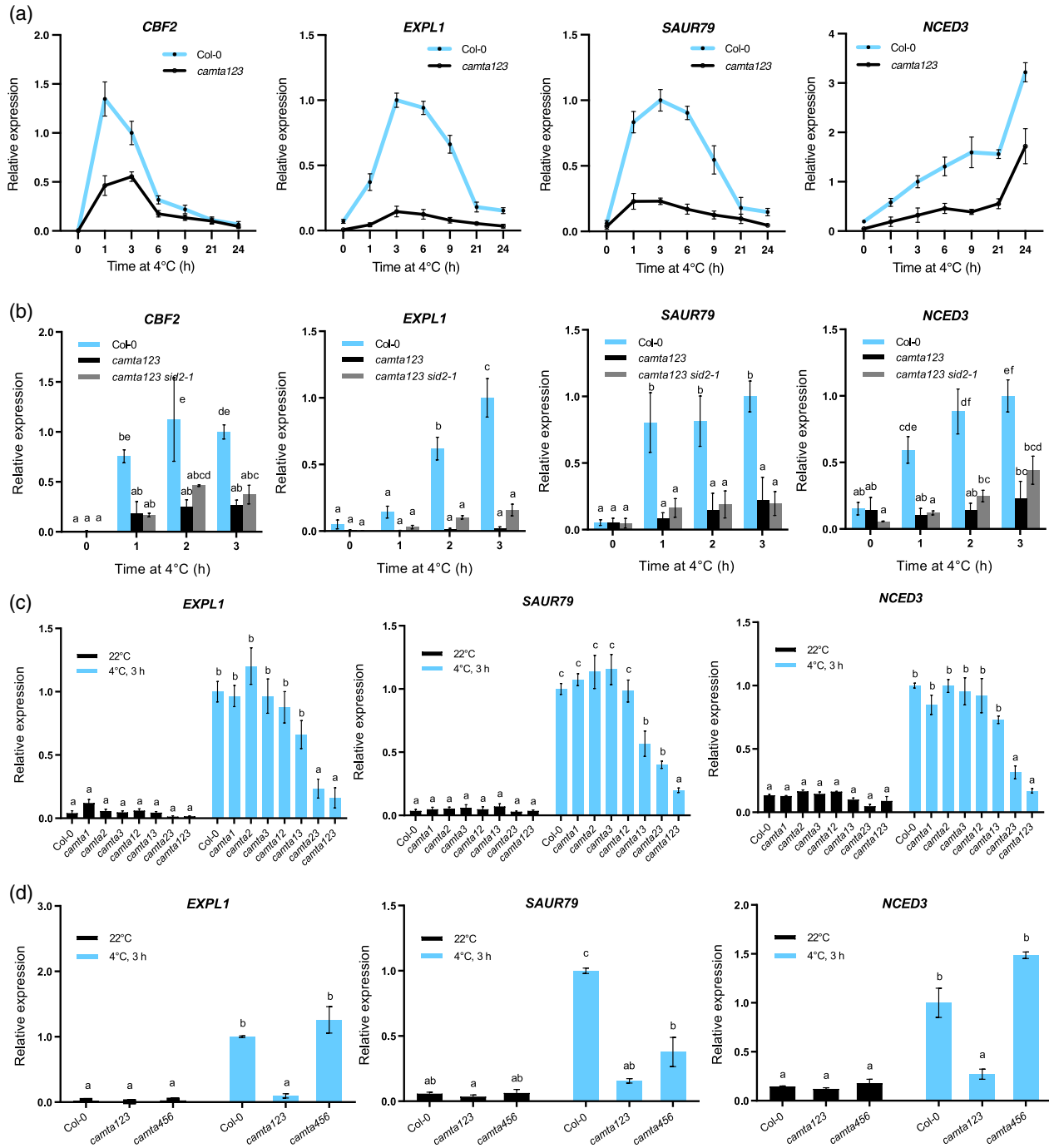
Kidokoro et al. (2017) showed that CAMTA5 contributes to the cold induction of *CBF1* and *CBF2*. To determine whether CAMTA5, or CAMTA4 and CAMTA6, contributes to the cold induction of *EXPL1*, *SAUR79* and *NCED3*, we investigated whether their induction was impaired in a *camta456* triple mutant. The results indicated that the induction of *SAUR79* was severely compromised in the *camta456* mutant, whereas the induction of *EXPL1* and *NCED3* was not (Figure 1d). These results were consistent with the findings of O'Malley et al. (2016) who tested the binding of CAMTA1 and CAMTA5 (as well as hundreds of other transcription factors) throughout the Arabidopsis genome and found that the promoters of *EXPL1* and *NCED3* bound CAMTA1, and that the promoter of *SAUR79* bound both CAMTA1 and CAMTA5. We therefore chose to focus our studies on *EXPL1* and *NCED3* as their regulation by CAMTAs was confined to CAMTA1, CAMTA2 and CAMTA3. Additionally, as little difference was observed between the cold induction of *EXPL1* and *NCED3* in the *camta23* and *camta123* triple mutants (Figure 1c), and that the *camta123* triple mutant is tiny in size and difficult to work with (Kim et al., 2013), we chose to use *camta23* as the genetic background in most of our experiments.

### The CAMTA3 NRM is sufficient to induce the expression of *CBF2*, *EXPL1* and *NCED3* in response to cold and to confer freezing tolerance

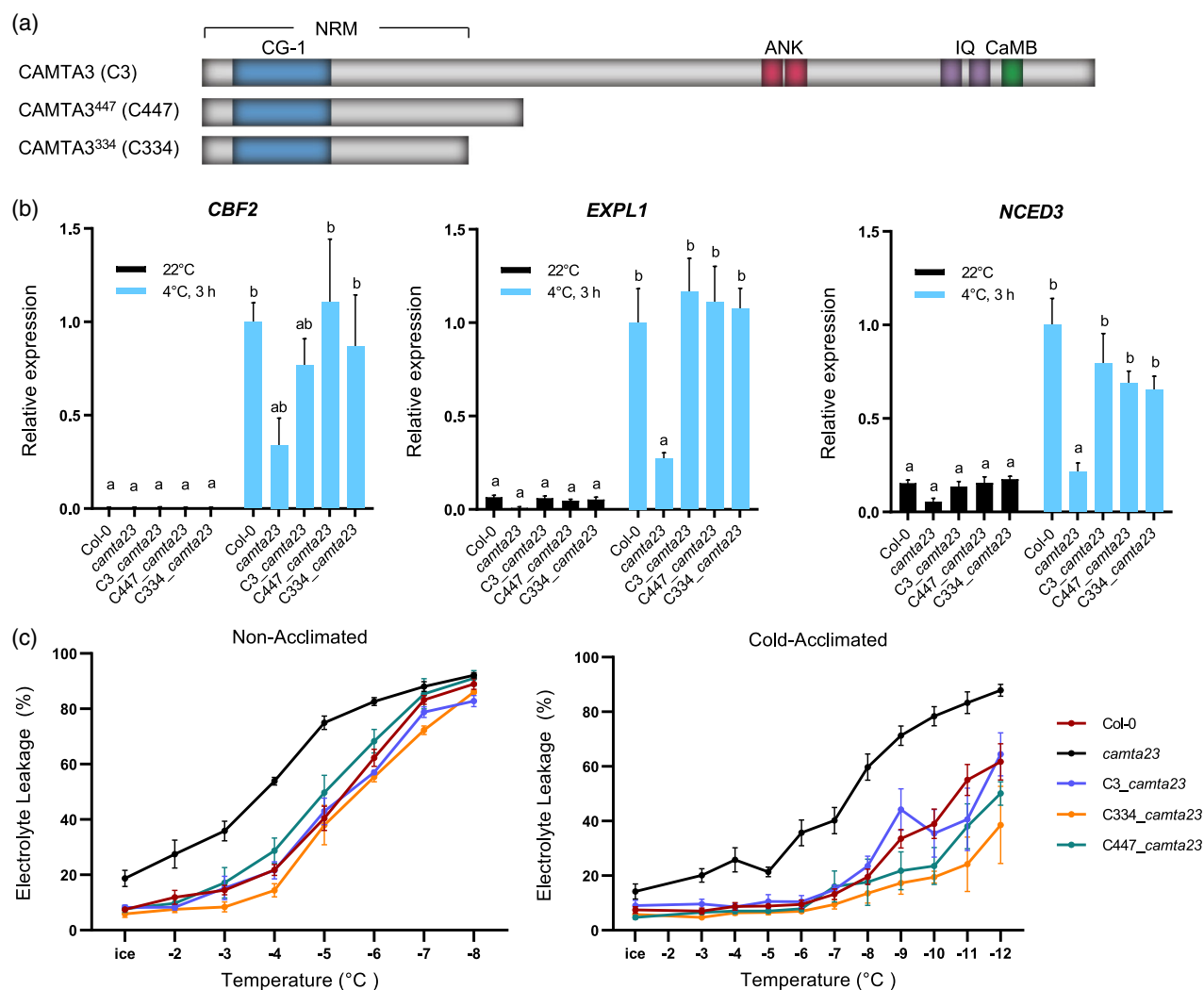
As noted above, we have previously shown that CAMTA3<sup>334</sup>, which includes the first 334 amino acids of CAMTA3, designated the NRM, was able to repress the expression of SA pathway genes in plants grown at moderate temperature (Kim et al., 2017). We were therefore interested in determining whether the CAMTA3 NRM was also capable of inducing the expression of target genes in response to cold temperature. Our results indicated that it was: we found that *CBF2*, *EXPL1* and *NCED3* were cold induced to the same degree in wild-type plants as they were in either *camta23* plants (Figure 2b) or *camta123* plants (Figure S1) expressing CAMTA3, CAMTA3<sup>334</sup> or CAMTA3<sup>447</sup> (Figure 2a) (CAMTA3 and its variants were tagged with GFP in these and all other experiments, unless indicated otherwise). Additionally, we found that the expression of CAMTA3<sup>334</sup> or CAMTA3<sup>447</sup> was as effective as expressing CAMTA3 in conditioning freezing tolerance in non-acclimated and cold-acclimated *camta23* plants (Figure 2c).

### CAMTA3 and CAMTA3<sup>334</sup> bind to target gene promoters at both moderate and cold temperatures

The rapid increase in *CBF2*, *EXPL1* and *NCED3* transcript levels that occurred in response to cold temperature (Figure 2b) could have been caused by a significant increase in CAMTA3 and CAMTA3<sup>334</sup> protein levels. This, however,



**Figure 1.** Rapid cold induction of *CBF2*, *EXPL1*, *SAUR79* and *NCED3* is impaired in the Arabidopsis *camta123* mutant. (a) Relative transcript levels of the indicated genes in Col-0 and *camta123* mutant plants upon exposure to cold temperature (4°C) for the indicated times. (b) Relative transcript levels of the indicated genes in Col-0, *camta123* and *camta123 sid2-1* plants upon exposure to cold temperature (4°C) for the indicated times. (c) Relative transcript levels of the indicated genes in Col-0 and *camta123* single, double and triple mutant plants grown at moderate temperature (22°C) and then exposed to cold temperature (4°C) for 3 h. (d) Relative transcript levels of the indicated genes in Col-0, *camta123* and *camta456* mutant plants grown at moderate temperature (22°C) and then exposed to cold temperature (4°C) for 3 h. Relative expression was normalized to the value obtained with Col-0 plants treated for 3 h at cold temperature (4°C), which was set to 1. Two-way ANOVA with a post-hoc Tukey HSD test was performed at  $P < 0.05$ . Different letters indicate significant differences.

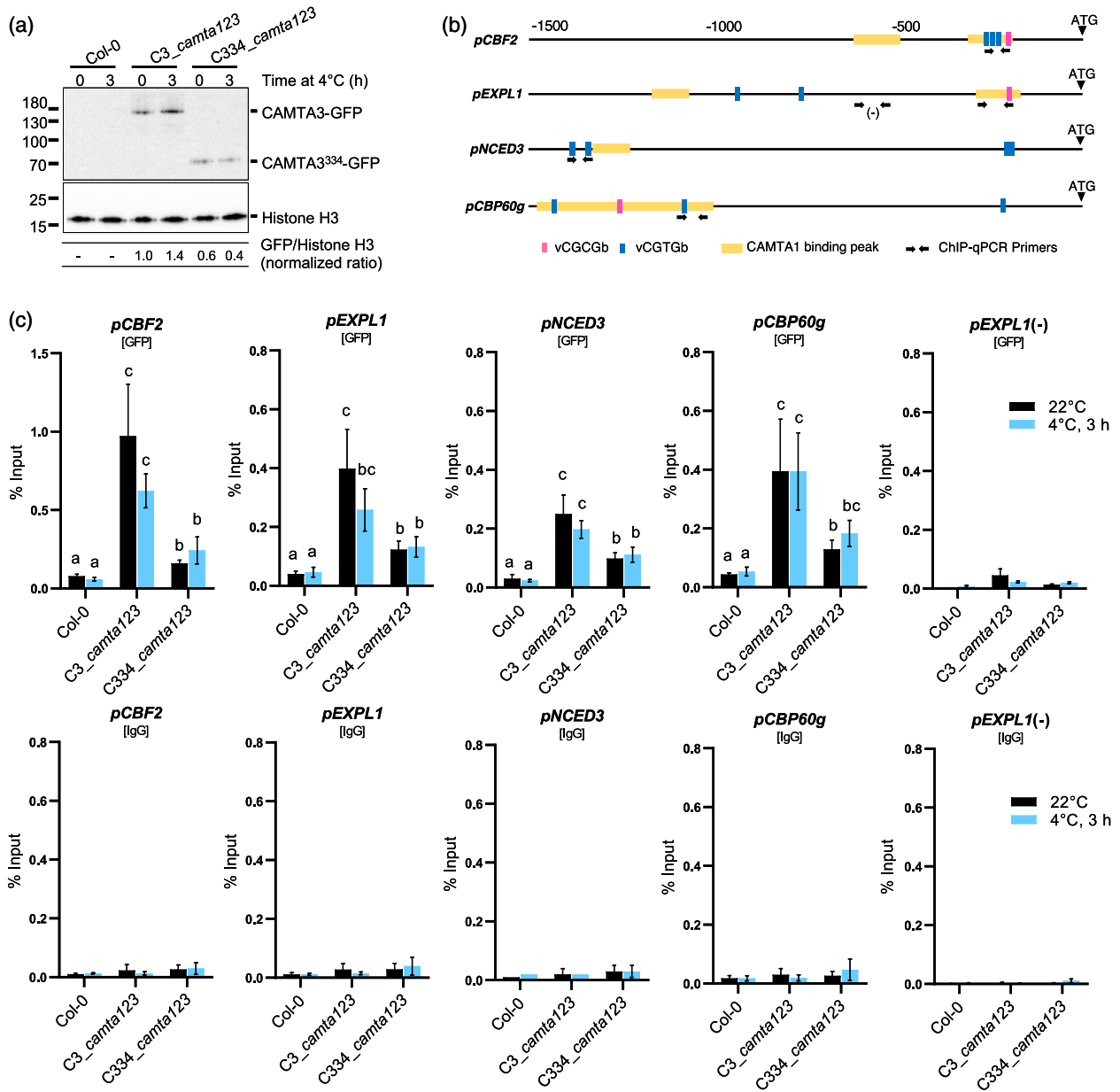


**Figure 2.** CAMTA3, CAMTA3<sup>334</sup> and CAMTA3<sup>447</sup> confer rapid cold induction of *CBF2*, *EXPL1* and *NCED3* and freezing tolerance in *camta23* mutant plants. (a) Diagram of CAMTA3, CAMTA3<sup>447</sup> and CAMTA3<sup>334</sup> proteins. The CG-1 DNA binding domain (CG-1), ankyrin repeat domain (ANK), IQ domains (IQ), calmodulin-binding domain (CaMB) and N-terminal repression module (NRM) are indicated. (b) Relative transcript levels of *CBF2*, *EXPL1* and *NCED3* in Col-0 and *camta23* plants and in *camta23* transgenic plants expressing CAMTA3, CAMTA3<sup>447</sup> and CAMTA3<sup>334</sup>. The plants were grown at moderate temperature (22°C) and then exposed to cold temperature (4°C) for 3 h. Relative expression was normalized to the value obtained with Col-0 plants treated for 3 h at cold temperature (4°C), which was set to 1. Two-way analysis of variance (ANOVA) with a post-hoc Tukey's honestly significant difference (HSD) test was performed at  $P < 0.05$ . Different letters indicate significant differences. (c) Electrolyte leakage freeze tests were conducted on plants grown at moderate temperature (22°C, non-acclimated) or exposed to cold temperature (4°C, cold-acclimated) for 3 weeks. Error bars are  $\pm$ SEMs of three biological replicates.

was not the case as immunoblot analysis indicated that the levels of nuclear-localized CAMTA3 and CAMTA3<sup>334</sup> protein increased only marginally, if at all, in response to cold treatment at 1 and 2 h (Figure S2) or 3 h (Figure 3a). Similarly, the levels of total protein for CAMTA3 and CAMTA3<sup>334</sup> were about the same in plants exposed to cold temperature for 0, 2 and 24 h (Figure S2).

Another possible explanation for the cold-induced increase in *CBF2*, *EXPL1* and *NCED3* transcript levels was that the binding of CAMTA3 and CAMTA3<sup>334</sup> to the promoters of these target genes could have been temperature dependent. To test this possibility, we conducted chromatin

immunoprecipitation quantitative real-time polymerase chain reaction (ChIP-qPCR) assays using *camta23* plants expressing either CAMTA3 or CAMTA3<sup>334</sup>. GFP antibody was used for the immunoprecipitation of the proteins and qPCR primers were used to amplify sequences located in regions of the promoters that included both a CAMTA DNA binding motif, vCGC/TGb, and a site shown by O'Malley et al. (2016) to bind CAMTA1 (Figure 3b). IgG antibody was used as a negative control for immunoprecipitation (Figure 3c) and a primer pair directed towards a region of the *EXPL1* promoter that did not include a CAMTA binding motif or the CAMTA1 binding site was used as a qPCR



**Figure 3.** CAMTA3 and CAMTA3<sup>334</sup> bind to target gene promoters at both moderate and cold temperatures.

(a) Protein levels of CAMTA3 and CAMTA3<sup>334</sup> in nuclei isolated from Col-0 and the *camta23* transgenic plants expressing either CAMTA3 (C3) or CAMTA3<sup>334</sup> (C334). Plants were grown at moderate temperature (22°C) and then exposed to cold temperature (4°C) for 3 h. Protein levels were determined by immunoblot analysis with anti-GFP antibody. Histone H3 was used as a loading control. The ratios of GFP/Histone H3 signals were determined with the 0 h value for CAMTA3 set to 1.0.

(b) CAMTA binding regions on the promoters of *CBF2*, *EXPL1*, *NCED3* and *CBP60g*. Blue and pink boxes indicate CAMTA binding the *cis*-elements vCGTGb and vCGCGb, respectively. Yellow boxes indicate the binding peaks of CAMTA1 as deposited in the Plant Cistrome Database (O'Malley et al., 2016). Black arrows indicate positions of promoter-specific primer pairs used in our ChIP-qPCR assays.

(c) ChIP-qPCR analysis of CAMTA3 or CAMTA3<sup>334</sup> binding to the promoter regions of the target genes. The chromatin from *camta23* transgenic plants expressing CAMTA3 (C3) and CAMTA3<sup>334</sup> (C334) grown at moderate temperature (22°C) and cold temperature (4°C) treated for 3 h were immunoprecipitated with GFP and IgG antibody, respectively, and the recovered DNAs were subjected to real-time qPCR with promoter-specific primer pairs. *pEXPL1(-)* was used as a negative control and *pCBP60g* was used as a positive control for CAMTA3 binding. Percentage input was calculated by  $2^{-\Delta\Delta Ct(GFP - Input)} \times 100$  from three biological replicates. Error bars indicate  $\pm$ SEMs of three independent biological replicates. Two-way analysis of variance (ANOVA) with Bonferroni test was performed with  $P < 0.05$ . Different letters indicate significant differences.

negative control (Figure 3b,c). The results indicated that both CAMTA3 and CAMTA3<sup>334</sup> bound to the promoters of *CBF2*, *EXPL1* and *NCED3* at moderate temperature and that

binding did not increase appreciably, if at all, in plants exposed to cold temperature for 1 and 2 h (Figure S3) or 3 h (Figure 3c). Thus, the dramatic cold-induced increase in

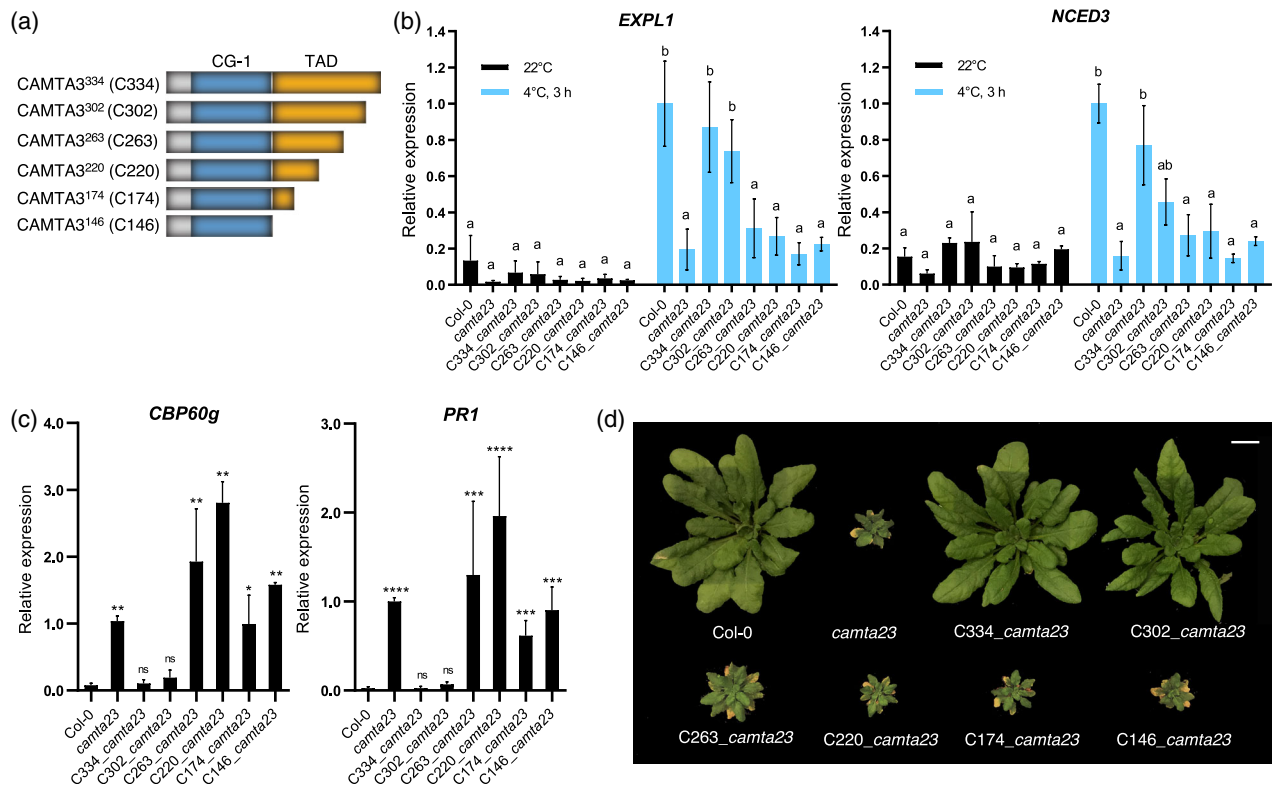
target gene expression mediated by CAMTA3 and CAMTA3<sup>334</sup> did not result from the temperature-dependent binding of the proteins to their target gene promoters.

One additional finding was that CAMTA3 and CAMTA3<sup>334</sup> bound to the promoter of *CBP60g* at about the same level in plants grown at moderate temperature or plants treated at cold temperature for 1 and 2 h (Figure S3) or 3 h (Figure 3c). These results were consistent with CAMTA3 and CAMTA3<sup>334</sup> repressing the expression of *CBP60g* in plants grown at moderate temperature and in plants treated at cold temperature for less than 1 week (Kim et al., 2013).

### The CAMTA3 TAD region is involved in both induction and repression of target gene expression

Our finding that CAMTA3<sup>334</sup> induced the expression of *EXPL1* and *NCED3* at cold temperature (Figure 2b)

indicated that this region included a TAD. This finding was consistent with the results of Bouché et al. (2002) who reported that a region of CAMTA1, just downstream of the CG-1 domain, could activate transcription in yeast. Similarly, we found that the region of CAMTA3 immediately downstream of the CG-1 domain (amino acids 147–334) was required and sufficient for stimulating transcription in yeast (Figure S4), and thus designated it the CAMTA3 TAD. To test whether the CAMTA3 TAD stimulated transcription in Arabidopsis, we created a deletion series that removed amino acids from the C-terminal end of CAMTA3<sup>334</sup> (Figure 4a) and determined whether the variants could induce the expression of *EXPL1* and *NCED3* in response to cold temperature (Figure 4b). The results showed that CAMTA3<sup>302</sup> was able to impart cold induction of *EXPL1* and *NCED3*, although perhaps not to the same degree as



**Figure 4.** The CAMTA3 TAD region is required for the cold induction of *EXPL1* and *NCED3*.

(a) Diagram of CAMTA3 variants. The amino acids included in the variants are indicated: CAMTA3<sup>334</sup> includes amino acids 1–344 of CAMTA3; CAMTA3<sup>302</sup> includes amino acids 1–302, etc.

(b) Relative transcript levels of *EXPL1* and *NCED3* in Col-0, *camta23* and the *camta23* transgenic plants expressing CAMTA3<sup>334</sup> (C334), CAMTA3<sup>302</sup> (C302), CAMTA3<sup>263</sup> (C263), CAMTA3<sup>220</sup> (C220), CAMTA3<sup>174</sup> (C174) and CAMTA3<sup>146</sup> (C146). Plants were grown at moderate temperature (22°C) and then exposed to cold temperature (4°C) for 3 h. Relative expression was normalized to the value obtained with Col-0 plants treated for 3 h at cold temperature (4°C), which was set to 1. Two-way analysis of variance (ANOVA) with a post-hoc Tukey's honestly significant difference (HSD) test was performed at  $P < 0.05$ . Different letters indicate significant differences.

(c) Relative transcript levels of *CBP60g* and *PR1* at moderate temperature (22°C) in Col-0, *camta23* and *camta23* transgenic plants expressing the indicated CAMTA3 variants as described in (b). Relative expression was normalized to the value obtained with *camta23* plants, which was set to 1. Data were log<sub>10</sub> transformed to reduce heteroscedasticity and analyzed by one-way ANOVA with Bonferroni's multiple comparison test at \*\*\*\* $P < 0.0001$ , \*\*\* $P < 0.001$ , \*\* $P < 0.01$  and \* $P < 0.05$ , not significant. Values shown in the figure are untransformed for clarity.

(d) Growth phenotypes of Col-0, *camta23* and *camta23* transgenic plants expressing the indicated CAMTA3 variants as described in (b) were grown at moderate temperature (22°C) for 42 days. Scale bar: 1 cm.

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The Plant Journal, (2022), 112, 235–248

CAMTA3<sup>334</sup>, and that CAMTA3<sup>263</sup>, CAMTA3<sup>220</sup>, CAMTA3<sup>174</sup> and CAMTA3<sup>146</sup> had little if any induction activity. This lack of activity was not linked to a lack of protein accumulation, as the levels of the protein variants tested were about the same or greater than the level of CAMTA3<sup>334</sup> in the transgenic lines treated at either moderate or cold temperatures (Figure S5). Taken together, these results indicate that sequences within the CAMTA3 TAD were essential for cold-induced expression of CAMTA3 target genes and that the amino acids between 263 and 302 were critical for this activity.

We previously showed that CAMTA3<sup>334</sup> represses the expression of *CBP60g*, which induces the expression of *ICS1*, resulting in SA biosynthesis, and *PR1*, which is induced by SA (Kim et al., 2017). We were interested to determine whether the CAMTA3 TAD sequences required for the cold induction of *EXPL1* and *NCED3* were also required for the repression of *CBP60g* and *PR1* at moderate temperature. We found that they were. Whereas the transcript levels for *CBP60g* and *PR1* were low in wild-type plants and in *camta23* plants expressing CAMTA3<sup>334</sup> or CAMTA3<sup>302</sup>, they were high in *camta23* plants and *camta23* plants expressing CAMTA3<sup>263</sup>, CAMTA3<sup>220</sup>, CAMTA3<sup>174</sup> and CAMTA3<sup>146</sup> (Figure 4c). Consistent with these results was that the small size of the *camta23* plants, which largely results from elevated SA levels (Kim et al., 2013), was complemented by the expression of CAMTA3<sup>334</sup> and CAMTA3<sup>302</sup>, but not by the expression of CAMTA3<sup>263</sup>, CAMTA3<sup>220</sup>, CAMTA3<sup>174</sup> or CAMTA3<sup>146</sup> (Figure 4d). Thus, the amino acids between 263 and 302 within the CAMTA3 TAD are also critical for repressing SA gene expression at moderate temperature.

### The CAMTA3 CG-1 domain confers temperature modulation of CAMTA3<sup>334</sup> induction activity

Our finding that CAMTA3<sup>334</sup> binds to the promoters of target genes at moderate temperature, but does not induce their transcription, suggested that the CAMTA3 TAD might only be active at cold temperature. However, protein fusion experiments indicated that this was not the case (Figure 5a,b). Specifically, we fused the CAMTA3 TAD region to the N-terminal region of CBF2 (amino acids 1–137) – a region that includes the CBF2 DNA binding domain (DBD), but lacks the CBF2 TAD (Wang et al., 2005) – and found that the hybrid protein, CBF2-DBD:CAMTA3-TAD (Figure 5a), expressed under control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter, induced the expression of *GOLS3* in a dose-dependent manner in plants grown at moderate temperature (Figure 5b); *GOLS3* is a target gene of CBF2, which is induced at moderate temperature in transgenic plants expressing the native CBF2 protein under control of the CaMV 35S promoter (Figure 5b; Park et al., 2015). In contrast to CBF2-DBD:CAMTA3-TAD, the overexpression of CAMTA3<sup>334</sup> or

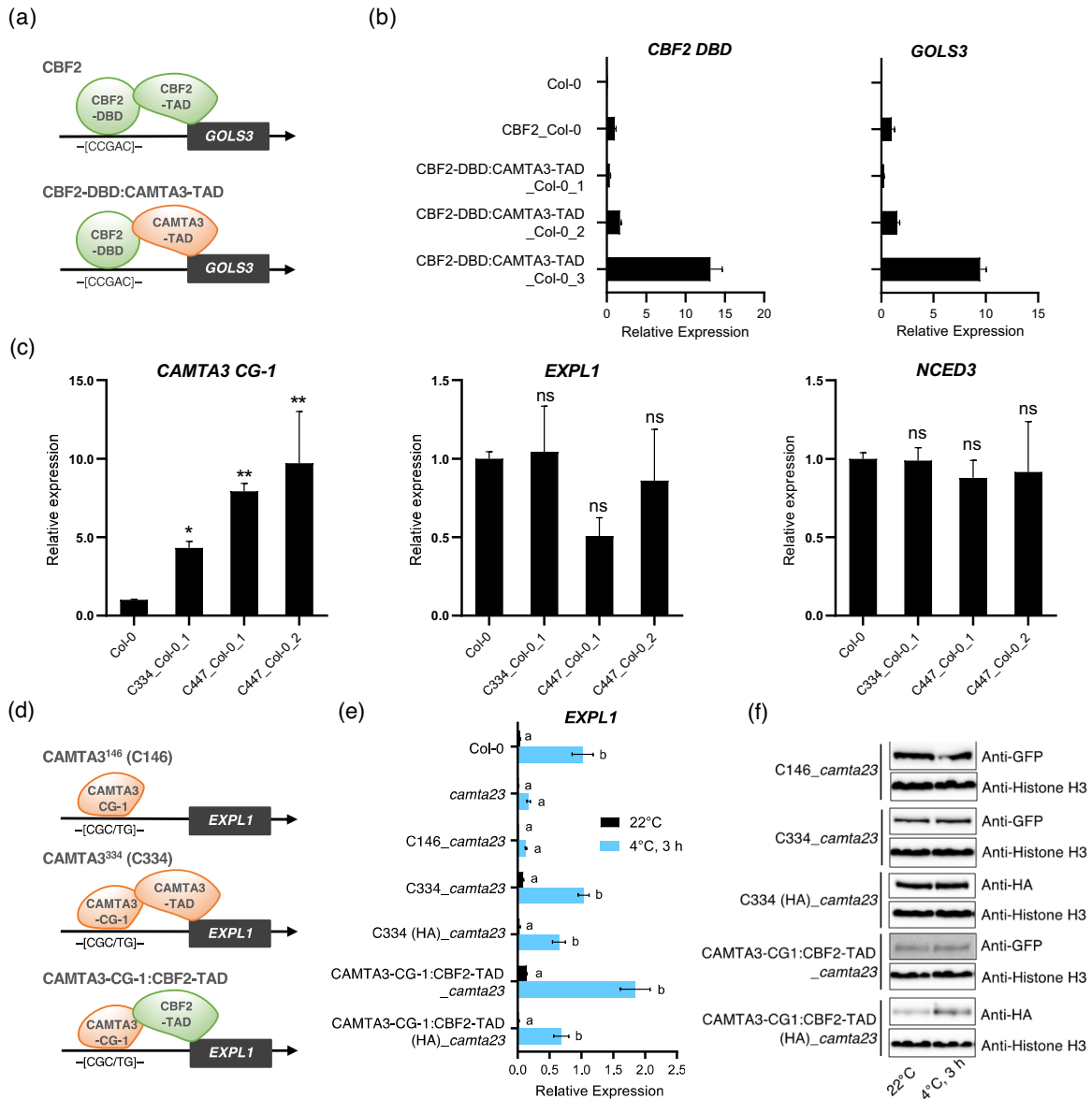
CAMTA3<sup>447</sup> under control of the CaMV 35S promoter did not induce the expression of the CAMTA3 target genes *EXPL1* or *NCED3* in plants grown at moderate temperature (Figure 5c). Taken together, these results indicate that the CAMTA3 TAD had the ability to induce transcription of target genes at moderate temperature when it was fused to the CBF2 DNA binding domain, but not when it was fused to the CAMTA3 CG-1 domain. Thus, it is possible that the CAMTA3 CG-1 domain imparts the temperature-dependent modulation of TAD activity.

To determine whether the CAMTA3 CG-1 domain had the ability to impart the temperature-dependent modulation of TAD activity, we fused it to the CBF2 TAD and investigated whether the hybrid protein, CAMTA3-CG-1:CBF2-TAD (Figure 5d), induced the expression of *EXPL1* in a temperature-dependent manner. The results indicated that it did: in plants expressing CAMTA3-CG-1:CBF2-TAD, as in those expressing CAMTA3<sup>334</sup>, *EXPL1* transcript levels were much greater (>10-fold) in plants exposed to cold temperature than they were in plants grown at moderate temperature (Figure 5e). This cold induction was not the result of changes in the expression of the transgenes, as the protein (Figure 5f) and transcript levels (Figure S6) for CAMTA3-CG-1:CBF2-TAD and CAMTA3<sup>334</sup> were essentially the same in plants treated at moderate and cold temperatures.

Like all of the CAMTA3 variant constructs tested in this study, the CAMTA3<sup>334</sup> and CAMTA3-CG-1:CBF2-TAD proteins were fused to GFP. To be certain that the GFP-tag did not impart the observed temperature-dependent induction of target genes, we fused CAMTA3<sup>334</sup> and CAMTA3-CG-1:CBF2-TAD to an HA-tag and determined whether they also induced the expression of *EXPL1* in a temperature-dependent manner. They did: in plants expressing HA-tagged CAMTA3<sup>334</sup> and CAMTA3-CG-1:CBF2-TAD, the transcript levels for *EXPL1* were much greater (>20-fold) in the plants treated at cold temperature than there were in plants grown at moderate temperature (Figure 5e). Again, this cold induction was not the result of changes in the expression of the transgenes, as the protein (Figure 5f) and transcript levels (Figure S6) for CAMTA3-CG-1:CBF2-TAD and CAMTA3<sup>334</sup> were about the same (differed by less than twofold) in plants treated at moderate and cold temperatures.

### The CAMTA3 CG-1 domain confers cycloheximide (CHX)-mediated modulation of CAMTA3<sup>334</sup> induction activity

We previously reported that *CBF2* and a number of other rapid ‘first-wave’ cold-responsive genes were induced in response to CHX treatment in plants grown at moderate temperature (Vogel et al., 2005). We found that this was also true for *EXPL1* (Figure 6). Further, we found that the CHX-mediated induction of *CBF2* and *EXPL1* was impaired in the *camta23* mutant and that induction was restored to a significant degree upon expressing CAMTA3<sup>334</sup> or



**Figure 5.** The CAMTA3 CG-1 domain suppresses the ability of the CAMTA3 and CBF2 TADs to induce gene expression at moderate temperature.

(a) Diagrams of CBF2 and CBF2-DBD:CAMTA3-TAD proteins used for the transcriptional activation of *GOLS3*.

(b) Relative transcript levels of the CBF2 and CBF2-DBD:CAMTA3-TAD transgenes expressed in Col-0 under control of the CaMV 35S promoter (left) and the CBF2 target gene *GOLS3* in Col-0 and the indicated transgenic lines (right). *CBF2* and *CBF2-DBD:CAMTA3-TAD* transcript levels were determined using *CBF2 DBD*-specific primers. Plants were grown at moderate temperature (22°C). Relative expression was normalized to the value obtained with the *CBF2\_Col-0* plants, which was set to 1.

(c) Relative transcript levels of the endogenous *CAMTA3* gene in Col-0 and the *CAMTA3*<sup>334</sup> and *CAMTA3*<sup>447</sup> transgenes expressed in Col-0 under control of the CaMV 35S promoter (left) and the *CAMTA3* target genes *EXPL1* and *NCED3* in the indicated transgenic lines (right two lanes). *CAMTA3* and transgene transcripts were detected with *CAMTA3 CG-1*-specific primers. Plants were grown at moderate temperature (22°C). Relative expression was normalized to the value obtained with Col-0 plants, which was set to 1. One-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test was performed at \*\**P* < 0.01 and \**P* < 0.05. ns, not significant.

(d) Diagrams of CAMTA3<sup>146</sup>, CAMTA3<sup>334</sup> and CAMTA3-CG-1:CBF2-TAD proteins.

(e) Relative transcript levels of *EXPL1* in Col-0, *camta23* and the *camta23* transgenic lines expressing the indicated proteins under the control of the CAMTA3 promoter. The proteins that were tagged with HA are indicated (HA); all other proteins were tagged with GFP. Plants were grown at moderate temperature (22°C) and transferred to cold temperature (4°C) for 3 h. Relative expression was normalized to the value obtained with Col-0 plants treated for 3 h at cold temperature (4°C), which was set to 1. Two-way ANOVA with a post-hoc Tukey's honestly significant difference (HSD) test was performed at *P* < 0.05. Different letters indicate significant differences.

(f) Protein levels of the indicated CAMTA3 variants. Total protein was extracted from *camta23* transgenic plants expressing CAMTA3<sup>146</sup> (C146), CAMTA3<sup>334</sup> (C334) or CAMTA3-CG-1:CBF2-TAD. Plants were grown at moderate temperature (22°C) and then exposed to cold temperature (4°C) for 3 h. Protein levels were determined by immunoblot analysis with anti-GFP or anti-HA antibody. Histone H3 was used as a loading control.

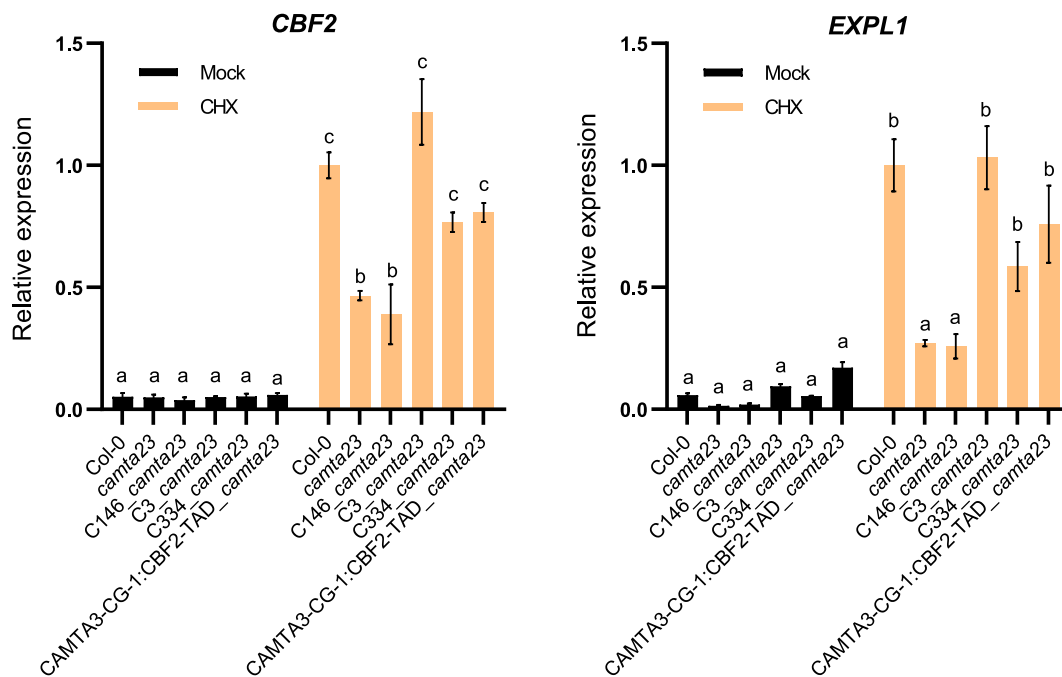
CAMTA3-CG-1:CBF2-TAD (Figure 6). These results indicated that the CHX-mediated induction of *CBF2* and *EXPL1* proceeded through a CAMTA pathway. Moreover, the fact that CHX induction was observed with fusion of the CAMTA3 CG-1 domain to either CAMTA3 TAD or CBF2 TAD indicated that CHX regulation proceeded, at least in part, through the CAMTA3 CG-1 domain. These results also provided additional evidence that the CAMTA3 TAD was capable of inducing gene expression at moderate temperature and that this activity was suppressed by the CAMTA3 CG-1 domain.

## DISCUSSION

Determining how plants sense low temperature and process the information to induce the expression of genes that impart freezing tolerance is a fundamental goal of cold acclimation research. Here we present results that provide insight into how the CAMTA3 transcription factor rapidly induces the expression of target genes in response to cold temperature. Two key observations were that the ability of CAMTA3 and CAMTA3<sup>334</sup> to induce the expression of *EXPL1*, *NCED3* and *CBF2* in response to cold temperature did not involve a significant increase in the levels of nuclear-localized CAMTA3 or CAMTA3<sup>334</sup> protein (Figures 3a and S2), and nor did it involve the cold-induced binding of CAMTA3 or CAMTA3<sup>334</sup> to target gene

promoters (Figures 3b,c and S3). Rather, our results indicate that the N-terminal CG-1 DNA binding domain imparts temperature-dependent modulation of CAMTA3 TAD activity (Figure 5). (An alternative mechanism has been proposed in a bioRxiv preprint posted by Guillaume-Schöpfer et al. (2020). These investigators present evidence from which they conclude that exposing plants to cold temperature results in CAMTA1, CAMTA2, and CAMTA3 binding to the promoters of target genes and inducing their expression.)

Our finding that CAMTA3<sup>334</sup> induced the expression of target genes in response to cold temperature indicated that the CAMTA3 NRM must include a TAD. Consistent with this was our finding that the region of CAMTA3 between amino acids 147 and 334 could activate transcription in yeast (Figure S4), and that the amino acids between 263 and 302 were required for rapid cold induction of *EXPL1* and *NCED3* in Arabidopsis (Figure 4a,b). The fact that the CAMTA3 NRM did not induce the expression of *EXPL1*, *NCED3* or *CBF2* at moderate temperature indicated that the ability of CAMTA3 TAD to induce gene expression was temperature dependent. However, this temperature dependence was not an intrinsic property of the CAMTA3 TAD as it was able to induce gene expression at moderate temperature when fused to the CBF2 DNA binding domain (Figure 5a,b). These results indicated that the CAMTA3 TAD



**Figure 6.** CAMTA3 mediates the cycloheximide (CHX) induction of *CBF2* and *EXPL1* in plants grown at moderate temperature.

Col-0, *camta23* and the *camta23* transgenic plants expressing CAMTA3<sup>146</sup> (C146), CAMTA3 (C3), CAMTA3<sup>334</sup> (C334) and CAMTA3-CG-1:CBF2-TAD were grown at moderate (22°C) temperature and then treated either with CHX (100 μM) or without (Mock) for 3 h. Relative transcript levels were normalized to the value obtained with Col-0 plants treated for 3 h with CHX, which was set to 1. Two-way analysis of variance (ANOVA) with a post-hoc Tukey's honestly significant difference (HSD) test was performed at  $P < 0.05$ . Different letters indicate significant differences.

sequence had the ability to induce gene expression at moderate temperature, but that this activity was suppressed when it was fused to the CAMTA3 CG-1 domain. Further support for this suppression model was our finding that the gene induction activity of the CBF2 TAD, which constitutively induces target gene expression when fused to the CBF2 DNA binding domain, was temperature dependent when fused to the CAMTA3 CG-1 domain (Figure 5c, d). Thus, in some manner, the CAMTA3 CG-1 region was able to suppress the ability of both the CAMTA3 TAD and the CBF2 TAD to induce gene expression at moderate temperature but not at cold temperature.

How might the CAMTA3 CG-1 domain impart temperature dependence on the ability of the CAMTA3 TAD to induce gene expression? In this regard, the results of our CHX experiments are of interest. We previously reported that *CBF2*, along with six other 'first-wave' genes that were rapidly induced in response to cold temperature, were induced in response to CHX treatment in plants grown at moderate temperature (Vogel et al., 2005). Further, analysis of the *CBF2* promoter provided evidence that the CHX induction of *CBF2* involved the action of CAMTA3 (Doherty et al., 2009). Our results presented here support this conclusion: both *CBF2* and *EXPL1* were induced in response to CHX treatment in plants grown at moderate temperature; this induction was impaired in the *camta23* mutant; and CHX induction of *CBF2* and *EXPL1* could be restored in the *camta23* mutant by expressing either CAMTA3 or CAMTA3<sup>334</sup> (Figure 6). These results indicate that CAMTA3 and CAMTA3<sup>334</sup> have the ability to induce the expression of target genes at moderate temperature, but that this activity is inhibited in non-stressed plants.

A simple model to explain the temperature modulation of CAMTA3 and CAMTA3<sup>334</sup> transcription induction activity is suggested by the fact that CHX inhibits protein synthesis. In this model, a suppressor protein, SP, that is rapidly turned over is synthesized in plants grown at moderate temperature, accumulates, binds to the CAMTA3 CG-1 domain, and blocks the ability of the CAMTA3 TAD to induce gene expression. SP is likely to bind to the CG-1 domain as the hybrid CAMTA3-CG-1:CBF2-TAD protein, which does not have the CAMTA3 TAD, also restored CHX induction of *CBF2* and *EXPL1* in the *camta23* mutant (Figure 6). Upon exposing plants to cold temperature, a condition that transiently reduces the rate of protein synthesis (Guillaume-Schöpfer et al., 2020; Wang et al., 2017), SP would become depleted (as presumably occurs in plants treated with CHX), thus allowing the CAMTA3 TAD to induce the expression of target genes. As the plants acclimate to cold temperature and protein synthesis increases, SP would again accumulate and inhibit CAMTA3 and CAMTA3<sup>334</sup> gene induction activity, thus accounting for the observed transient cold induction of the target genes (Figure 1a).

Of course, temperature modulation of CAMTA3 and CAMTA3<sup>334</sup> transcription induction activity could occur through many other mechanisms. One mechanism, a modification of the SP suppression model just presented, would be that SP does not bind to the CAMTA3 CG-1 domain, but rather binds to a *cis*-acting regulatory element linked to the CG-1 *cis*-acting regulatory element and in some way – perhaps through chromatin remodeling – negates CAMTA3 transcriptional activation activity. Again, the level of SP would transiently decrease in response to cold temperature and allow for the transient induction of CAMTA3 target genes. Other possibilities would include mechanisms suggested by Benn et al. (2014) who established that CAMTA proteins, including CAMTA3, play a role in the general stress response in Arabidopsis, including the induction of genes in response to wounding (Benn et al., 2014; Walley et al., 2007). As discussed by the investigators, the modulation of CAMTA3 activity could occur through many pathways, such as post-translational modification of CAMTA3 itself or the modification of proteins that affect CAMTA activity.

As for cold temperature generating a signal that affects the ability of CAMTA3 to induce gene expression, our finding that CAMTA3<sup>334</sup> confers the rapid cold induction of target genes was unexpected. This is because the finding was at odds with our previous suggestion (Doherty et al., 2009) that the rapid influx of Ca<sup>2+</sup> into the cytoplasm that occurs upon exposing plants to cold temperature (Knight et al., 1991) leads to the formation of Ca<sup>2+</sup>-calmodulin, which then binds to CAMTA3 and in some way enables it to induce gene expression. The fact that CAMTA3<sup>334</sup> does not include the two domains of CAMTA3 known to bind Ca<sup>2+</sup>-calmodulin, namely the IQ and CaMB domains, provides evidence that Ca<sup>2+</sup>-calmodulin binding to CAMTA3 is not required for induction activity. Although we think that this is the most likely situation, there are two formal possibilities to consider: perhaps CAMTA3<sup>334</sup> includes a novel Ca<sup>2+</sup>-calmodulin binding domain or Ca<sup>2+</sup>-calmodulin binding to CAMTA3 is required in the context of the entire CAMTA3 protein. A more significant possibility, however, is that the binding of Ca<sup>2+</sup>-calmodulin to CAMTA3 is *not* required for the rapid induction of target genes in response to cold temperature but *is* required for suppressing the ability of CAMTA3 to repress the expression of SA-mediated immunity genes in response to pathogen recognition and long-term exposure of plants to cold temperature (Du et al., 2009; Kim et al., 2013). These and other possibilities must now be tested.

A final point regards the dual role of CAMTA3 as an inducer of freezing tolerance genes and repressor of SA-mediated immunity genes. Here we show that the CAMTA3 TAD region between amino acids 263 and 302 is required for both the induction of *EXPL1* and *NCED3* at cold temperature and the repression of *CBP60g* at moderate

temperature (Figure 4). This finding provides evidence that the transcriptional activation and repression activities of CAMTA3 are physically linked. Thus, an important area of future study is determining the mechanism by which the CAMTA3 TAD region induces the expression of some target genes at cold temperature and represses the expression of others at moderate temperature.

## EXPERIMENTAL PROCEDURES

### Plant materials and growth conditions

All *A. thaliana* plants used in this study were in the Col-0 background. The *camta1* (SALK\_008187), *camta2* (SALK\_007027) and *camta3* (SALK\_001152), *camta1/2*, *camta1/3*, *camta2/3*, *camta1 camta2 camta3 (camta123)* and *camta123 sid2-1* mutants were described previously (Doherty et al., 2009; Kim et al., 2013; Kim et al., 2020). The *camta456* triple mutant was generated by crossing *camta4* (Salk\_013723), *camta5* (GABI-Kat Line GK-815B08) and *camta6* (Salk\_078900). Seeds were stratified in the dark at 4°C for 2–3 days before germination. All plants were grown in soil in a growth chamber at 22°C using a 12-h photoperiod with a light intensity of approximately 120  $\mu\text{mol m}^{-2} \text{sec}^{-1}$ . Aerial parts of the plants were analyzed in all experiments unless indicated otherwise. Biological replicates were from independent experiments.

### Vector constructions and plant transformations

The *camta23* transgenic plants expressing CAMTA3 (C3), CAMTA3<sup>334</sup> (C334) and CAMTA3<sup>447</sup> (C447) have been described previously (Kim et al., 2017). The binary gateway vector pEGC3PGFP (Kim et al., 2017) was used to insert CAMTA3<sup>302</sup> (C302), CAMTA3<sup>263</sup> (C263), CAMTA3<sup>220</sup> (C220), CAMTA3<sup>174</sup> (C174) and CAMTA3<sup>146</sup> (C146) under the control of the native CAMTA3 promoter. The N-terminal region of CBF2 (1–136), which contains its DNA binding domain, was fused to CAMTA3 TAD (147–334) by overlap extension PCR (Lee et al., 2010). The hybrid protein CBF2-DBD:CAMTA3-TAD was cloned into the pEarleyGate100 vector. The N-terminal region of CAMTA3 (1–146), which contains the CG-1 domain, was fused to the C-terminal region of CBF2 (137–216), which contains its TAD, by overlap extension PCR. The hybrid protein CAMTA3-CG-1:CBF2-TAD was cloned into pEGC3PGFP. The hemagglutinin (HA)-tagged binary gateway vector pEGC3PHA, generated by inserting 2 kb of the CAMTA3 upstream region into pEarleyGate301, was used to produce CAMTA3<sup>334</sup> (HA) and CAMTA3-CG-1:CBF2-TAD (HA) expressing vectors. Constructs were introduced into *camta23* or *camta123* by the floral-dip transformation method (Clough & Bent, 1998), and homozygous T<sub>3</sub> (or higher) generation plants were used in this study.

### Cold and CHX treatment

Plants grown for 21 days at 22°C were subjected to cold treatment at Zeitgeber time 4 (ZT4; 4 h after dawn). Cold treatment was performed at 4°C for the indicated time under a 12-h photoperiod with a light intensity of approximately 35  $\mu\text{mol m}^{-2} \text{sec}^{-1}$ . The aerial parts of the plants were harvested by flash freezing in liquid nitrogen after treatment for the indicated time. For the CHX treatment, seedlings were grown under continuous white light on solidified half-strength Gamborg's medium for 7 days after a 2-day imbibition at 4°C. The seedlings were transferred to liquid half-strength Gamborg's medium with or without 100  $\mu\text{M}$  CHX and were harvested after a 3 h of treatment by briefly drying on tissue paper and flash freezing in liquid nitrogen.

### Freezing tolerance tests

Electrolyte leakage assays for freezing tolerance were performed as described previously (Park et al., 2015). Plants were grown and subjected to cold treatment under a 12-h photoperiod.

### Transactivation assay in yeast

To determine the CAMTA3 transcriptional activation domain, CAMTA3 and its deletion variants  $\Delta\text{IQ}$  (1–850),  $\Delta\text{N146}$  (147–1032),  $\Delta\text{N334}$  (335–1032) and TAD (147–334) were cloned into pDEST32 to produce yeast expression vectors with GAL4 DNA binding domain (DB) fused to CAMTA3 or its deletion variants. The resulting plasmids were introduced into *Saccharomyces cerevisiae* AH109, in which the *HIS3* reporter gene was driven by the GAL4 promoter. Each transformant was selected by growth on LEU-dropout synthetic medium and tested by growth on His-dropout synthetic medium (SD –His) supplemented with 3-amino-1,2,3-triazole (3-AT) for high stringency conditions. The growth of cells was photographed at 3 or 7 days after spotting onto SD –Leu/–His media.

### Quantification of transcript levels

Total RNA was extracted using an RNeasy Plant Mini Kit (cat. no. 74904; Qiagen, <https://www.qiagen.com>) with an additional DNase treatment (cat. no. 79251; Qiagen). Total RNA (approx. 200 ng) was used to synthesize cDNA with random oligonucleotides using a reverse transcription system (cat. no. A3500; Promega, <https://www.promega.com>) according to the manufacturer's instructions using a 20- $\mu\text{l}$  reaction volume and an incubation time of 30 min at 42°C. The cDNA reaction mixture was diluted fivefold with water, and 2  $\mu\text{l}$  was used as a template in a 10- $\mu\text{l}$  PCR reaction using Applied Biosystems FAST 7500 real-time PCR system in standard mode with Fast SYBR Green Master Mix (cat. no. 4385612; Applied Biosystems, now ThermoFisher Scientific, <https://www.thermofisher.com>) according to the manufacturer's protocols. Reactions were performed in triplicate and products were checked by melting curve analysis. Primer sequences are given in Table S1. The abundance of the indicated transcripts was analyzed with the relative standard curve method normalizing to the reference transcript *PP2A*, unless indicated otherwise. Unless indicated otherwise, all quantitative reverse transcription PCR (qRT-PCR) replicates refer to three independent biological replicates and the mean  $\pm$  SEM of three independent biological replicates are plotted.

### Protein extraction and immunoblots

Leaf samples (100–200 mg) were ground in liquid nitrogen, heated in 2X NuPAGE™ LDS Sample Buffer (1:3) for 10 min at 95°C, and then centrifuged. Protein samples were analyzed on a 4%–12% BT NuPAGE gel (cat. no. NP0321BOX; Life Technologies, now ThermoFisher Scientific, <https://www.thermofisher.com>) and proteins were transferred to nitrocellulose membrane (cat. no. 88018; Life Technologies, now ThermoFisher Scientific). The membrane was blocked with 5% skimmed milk in 1X Tris-buffered saline with Tween-20 (TBST) for 1 h at room temperature (21–25°C) followed by incubation with the primary antibody anti-GFP (11 814 460 001; Roche, available through Milliporesigma, <https://www.sigmaaldrich.com>), anti-HA-HRP (11 667 475 001, Roche) or anti-Histone H3 (07–690; Milliporesigma, <https://www.sigmaaldrich.com>) in 1% skimmed milk at 4°C overnight. The secondary antibody was horseradish peroxidase-conjugated anti-mouse (cat. no. 32430; ThermoFisher Scientific) or anti-rabbit IgG (cat. no. 32460, ThermoFisher Scientific). SuperSignal West Femto Maximum Sensitivity Substrate (P134095; FisherScientific, <https://www.fishersci.com>) was used for the visualization of

anti-GFP and anti-HA signal and SuperSignal™ West Pico PLUS Chemiluminescent Substrate (P134577; FisherScientific, <https://www.fishersci.com>) was used for anti-Histone H3 signal.

### ChIP-qPCR assay

Plants grown in soil at 22°C under a 12-h photoperiod for 3 weeks were treated at 4°C for 0, 1, 2 or 3 h. Harvested leaf tissues were cross-linked in fix-solution containing 1% formaldehyde and 400 mM sucrose with vacuum application. After the rinse, leaf samples were weighed and kept at -80°C for further analysis. About 2 g of leaf samples were ground in liquid nitrogen and resuspended in 5 ml of lysis buffer (20 mM Tris/HCl, 25% glycerol, 20 mM KCl, 2 mM EDTA, 2.5 mM MgCl<sub>2</sub>, 250 mM sucrose, 1× protease inhibitor cocktail, 1 mM PMSF, 10 mM β-mercaptoethanol, 50 μM MG-132) for homogenization. The lysate was filtered through two-layered Miracloth and centrifuged at 1500 g for 10 min at 4°C. The pellets were resuspended with 5 ml of NRBT buffer (20 mM Tris/HCl, 25% glycerol, 2.5 mM MgCl<sub>2</sub>, 0.5% Triton X-100, 1× protease inhibitor cocktail, 1 mM PMSF, 10 mM β-mercaptoethanol, 50 μM MG-132) and centrifuged at 1500 g for 10 min at 4°C three times and 3 ml of NRB buffer (20 mM Tris/HCl, 25% glycerol, 2.5 mM MgCl<sub>2</sub>, 1× protease inhibitor cocktail, 1 mM PMSF, 10 mM β-mercaptoethanol, 50 μM MG-132) and centrifuged once (Xu & Copeland, 2012). For chromatin immunoprecipitation, the manufacturer's protocol was followed (Pierce Magnetic ChIP Kit; ThermoFisher Scientific). The extracted nuclei were resuspended in 200 μl of 1× IP dilution buffer (20 mM Tris/HCl, 2 mM EDTA, 150 mM NaCl, 0.5% Triton X-100, 1× Halt protease/phosphatase inhibitors) and subjected to sonication at 20% output six times for 20 sec with 1-min intervals on ice. The supernatant containing sheared chromatin was recovered by centrifugation at 9000 g for 5 min. A 10-μl volume of chromatin was saved as an input. A 100-μl sample of chromatin was added to 400 μl of 1× IP dilution buffer and 10 μg of anti-GFP Ab and mixed at 4°C with rotation overnight. A 20-μl volume of Protein A/G magnetic beads was added and incubated for 2 h at 4°C with rotation. The chromatin-bound beads were washed with wash buffer 1 (20 mM Tris/HCl, 2 mM EDTA, 150 mM NaCl, 0.5% Triton X-100) three times and wash buffer 2 (20 mM Tris/HCl, 2 mM EDTA, 350 mM NaCl, 0.5% Triton X-100) once. Immunoprecipitated chromatin was eluted with 1× elution buffer (50 mM Tris/HCl, 10 mM EDTA, 1% SDS) at 65°C for 30 min and treated with Proteinase K at 65°C for 1.5 h. Eluted genomic DNA was recovered with a PCR purification kit (Qiagen) and diluted with nuclease-free water for qPCR analysis with promoter-specific primer pairs. *pEXPL1(-)* was used as a negative control and *pCBP60g* was used as a positive control for CAMTA3 binding.

### ACCESSION NUMBERS

The sequence data for genes presented in this article can be found in The Arabidopsis Information Resource (TAIR) database under the following accession numbers: *CAMTA1* (AT5G09410), *CAMTA2* (AT5G64220), *CAMTA3* (AT2G22300), *CAMTA4* (At1g67310), *CAMTA5* (At4g16150), *CAMTA6* (At3g16940), *CBF2* (At4g25470), *GOLS3* (At1g09350), *EXPL1* (AT3G45970), *NCED3* (AT3G14440), *SAUR79* (AT2G35290), *CBP60g* (AT5G26920), *PR1* (AT2G14610) and *PP2A* (AT5G08290).

### ACKNOWLEDGEMENTS

We thank Philip Wigge (Leibniz-Institut für Gemüse-und Zierpflanzenbau) for informative discussions regarding CAMTA-

mediated regulation of cold-inducible genes. This work was supported by grants to MFT from Michigan AgBioResearch (MICL02415) and the Michigan State University Foundation and infrastructure support from the Chemical Sciences, Geosciences, and Biosciences Division, Office of Basic Energy Sciences, US Department of Energy (DE-FG02-91ER20021).

### AUTHOR CONTRIBUTIONS

LC, YK and MFT conceptualized the project. LC, YK and SJG performed the experiments and the data analysis. LC and MFT wrote the article with input from YK and SJG.

### CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest associated with this work.

### DATA AVAILABILITY STATEMENT

Materials generated in this study will be made available upon request.

### SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

**Figure S1.** CAMTA3 and CAMTA3<sup>334</sup> rapidly induce *CBF2*, *EXPL1* and *NCED3* in response to cold temperature.

**Figure S2.** Protein levels of CAMTA3 or CAMTA3<sup>334</sup> in transgenic plants.

**Figure S3.** CAMTA3 and CAMTA3<sup>334</sup> bind to target gene promoters at both moderate and cold temperatures.

**Figure S4.** Identification of a region of CAMTA3 that serves as a transcriptional activation domain (TAD) in yeast.

**Figure S5.** Immunoblot analysis of CAMTA3 variants.

**Figure S6.** Transcript levels of *CAMTA*<sup>146</sup>, *CAMTA*<sup>334</sup> and *CAMTA3-CG-1:CBF2-TAD* at moderate and cold temperatures.

**Table S1.** Primers used for qRT-PCR in this study.

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