Different cold-signaling pathways function in the responses to rapid and gradual decreases in temperature

Satoshi Kidokoro, Koshi Yoneda, Hironori Takasaki, Fuminori Takahashi, Kazuo Shinozaki, and Kazuko Yamaguchi-Shinozaki

Plant Cell. Advance Publication March 28, 2017; doi: 10.1105/tpc.16.00669

Corresponding author: Kazuko Yamaguchi-Shinozaki, akys@mail.ecc.u-tokyo.ac.jp.

Review timeline:

TPC2016-00669-RA	Submission received:	Aug. 24, 2016
	1st Decision:	Sept. 26, 2016 *revision requested*
TPC2016-00669-RAR1	1st Revision received:	Dec. 11, 2016
	2nd Decision:	Feb. 2, 2017 *accept with minor revision*
TPC2016-00669-RAR2	2nd Revision received:	Feb. 19, 2017
	3rd Decision:	Mar. 9, 2017 *acceptance pending, sent to science editor*
	Final acceptance:	Mar. 22, 2017
	Advance publication:	Mar. 28, 2017

REPORT: (The report shows the major requests for revision and author responses. Minor comments for revision and miscellaneous correspondence are not included. The original format may not be reflected in this compilation, but the reviewer comments and author responses are not edited, except to correct minor typographical or spelling errors that could be a source of ambiguity.)

TPC2016-00669-RA 1st Editorial decision – *revision requested* Sept. 26, 2016

The reviewers and editorial board members consulted see the potential novelty in the manuscript and acknowledge the substantive amount of research undertaken thus far. However, there were some real concerns about the degree of confirmation about the existence of two pathways and that the "clock experiments" were not designed in such a way to test the mechanism and involvement of the clock - all you really demonstrated was that expression was circadian regulated and that this peak was suppressed in the mutants. There were also a number of specific questions and concerns about experimental design and statistical analysis in a range of the figures. On the positive side it is the opinion of the reviewers and two of the editors that you could address all these concerns in 3-4 months. At that stage we would request the same reviewers assess the manuscript and they and the editors will determine if you do satisfactorily address the concerns. Thus, it would be a full review and it would be up to the authors to ensure the major and minor concerns are addressed.

We ask you to pay attention to the following points in preparing your revision.

1. Better demonstration of the existence of two pathways. We would suggest more thought applied to experimental design than the simple rapid and stepped experimental experiments and showing that there are two sets of pathways, not a slow and fast response via the same pathway.

2. Demonstration of the involvement of the clock. We recommend you consult with experts in the clock to assist in experimental design and what each experiment enables you to conclude.

3. Improvements to text, figures and analyses. You will note there was a range of specific concerns about the figures and data analysis. Please read these carefully and correct and re-do experiments, statistical analyses and

presentation as necessary. For example, for some of the statistical analysis the real point of comparison is between higher order mutants than between mutants and wild type.

4. The array analyses as presented do not aid in interpretation. Please give them some thought and re-do the analyses to better test the central claims of the paper. Give consideration to different and broader analyses that enable more hypothesis testing. Is the regulation of the CAMTAs dependent or independent of the ICE pathway for example, can you use test the role of the clock in your array analyses?

5. Finally, please place more emphasis on what is novel, not on what is an extension of previously reported findings.

Please contact us if there are ambiguous comments or if you wish to discuss the revision.

---------- Reviewer comments:

[Reviewer comments shown below along with author responses]

TPC2016-00669-RAR1 1st Revision received Dec. 11, 2016

Reviewer comments and **author responses**:

RESPONSE: Thank you very much for your consideration of our manuscript entitled "Differential signaling in cold responses to rapid and gradual temperature decreases in Arabidopsis". We have revised our manuscript according to your and the reviewers' comments.

1. We performed statistical analyses on all qRT-PCR data (Figures 1A, 3C and 3D, 4B and 4C, 5B, and 6 and Supplemental Figures 8-11).

2. We performed new experiments to assess the effects of circadian rhythm on cold-inducible expression of the *DREB1* genes using plants transferred to free-running conditions under continuous light (Figure 6 and Supplemental Figure 11).

3. We performed new experiments to assess the effects of cooling rates and growth conditions on cold-inducible expression of the *DREB1A* and *DREB1B* genes using *camta3*, *camta5* and *camta4* single mutants (Supplemental Figure 8).

4. We analyzed the expression of *PR1*, an SA-induced gene, and *XTH31,* an SA-repressed gene, in WT and *camta* mutant plants (Figures 3D and 4C).

5. We revised the Discussion section to emphasize the novel aspects of the paper (P. 19, lines 376-400).

6. We analyzed expression of the *CAMTA* genes in WT plants grown on agar plates and in soil pots (Supplemental Figure 7).

7. We compared the down-regulated genes in *camta* mutant plants with the up-regulated genes in Arabidopsis plants overexpressing *DREB1B* (Supplemental Figure 6A).

8. We performed EMSA using a GST fusion protein of the DNA-binding domain of CAMTA3 (Supplemental Figure 4).

9. We detected DAPI-stained nuclei in plants expressing CAMTA-GFP driven by the CaMV *35S* promoter (Supplemental Figure 2).

10. We measured the temperature around plants grown on agar plates with or without lids (Supplemental Figure 9A).

11. We added the results of yeast one-hybrid screens (Supplemental Table 1).

Editor's comments:

Demonstration of the existence of two pathways. At the present time we cannot distinguish whether there is a slow and fast response via the same pathway or whether there are two pathways.

RESPONSE: As previously reported, Arabidopsis DREB1A, DREB1B, and DREB1C are transcriptional activators that function as main switches of low-temperature-responsive gene expression. These three transcription factors regulate many common cold-inducible genes. It has also been shown that these three *DREB1* genes themselves are strongly induced by cold stress. Therefore, induction of the expression of *DREB1s* is thought to be the first step in the cold-responsive transcriptional cascade. In this study, we have clearly demonstrated that the expression mechanisms of these three genes are different using *camta35*, *camta123456*, and *cca1/lhy* mutant plants. *DREB1B* is regulated by CAMTA3 and CAMTA5, but *DREB1A* is regulated by clock elements, such as CCA1 and LHY. As shown by the data obtained using mutant plants that are presented in the revised version of Figure 6, decreased *DREB1A* expression was observed only in the *cca1/lhy* mutant, and decreased *DREB1B* expression was observed only in the *camta* mutants. Thus, it seems that CAMTA and clock factors, such as CCA1 and LHY, participate in different signaling pathways, as shown in Figure 7. In addition, as shown in Figure 5, *DREB1A,* regulated by clock factors, responded to both rapid and slow temperature decreases, but *DREB1B,* regulated by CAMTAs, responded to only a rapid decrease in temperature, indicating that the activation mechanisms of the two pathways seem to be different. We discussed this point in the Discussion section (P. 19, lines 376-400).

Demonstration of the involvement of the clock. Perhaps experts in the clock can advise you concerning experimental design and the conclusions that can be drawn from each experiment.

RESPONSE: We obtained advice concerning the experimental design and conclusions that can be drawn from each experiment from a circadian clock expert, according to the Editor's suggestion (P. 27, lines 613–615; Acknowledgements). With regard to the revised version of Figure 6 and Supplemental Figure 11, which are related to circadian rhythm, we performed expression analyses using plants transferred to free-running conditions under continuous light, according to the expert's suggestion. The results revealed that *DREB1A* and *DREB1C* expression was affected by circadian rhythm, indicating involvement of the clock in the expression mechanisms of these genes. We revised the text concerning the experiments according to the expert's suggestion (P. 17, line 337 - P. 18, line 367).

Improvements to text, figures and analyses. Reviewers have mentioned specific issues about individual figures and about data analysis. Please read these comments carefully and either correct the presentation or re-do the experiments, as appropriate, and provide statistical analyses as necessary. For example, for some of the statistical analysis the relevant comparison is between higher order mutants than between mutants and wild-type. The replicates must be described in the figure legends, whether they are from independent experiments, plants, samples, etc.

RESPONSE: We performed several experiments (Figures 3D, 4C, and 6 and Supplemental Figures 2, 4, 7, 8 and 11A) and statistical analyses (Figures 1A, 3C and 3D, 4B and 4C, 5B, and 6 and Supplemental Figures 8, 9B, 10, and 11), and the text and figure legends were revised according to the suggestions of the editor and reviewers. These revisions are presented point-by-point in the "Responses to the reviewers' comments".

The array analyses as presented do not provide information to the reader. Please give them some thought and re-do the analyses to better test the central claims of the paper. Give consideration to different and broader analyses that enable more hypothesis testing. Is the regulation of the CAMTAs dependent or independent of the ICE pathway for example, can you use test the role of the clock in your array analyses?

RESPONSE: According to the reviewer's suggestion, we moved the RNA-seq data from Figure 5 to Supplemental Figure 6. We also compared the down-regulated genes in the *camta* mutant with the cold-inducible genes and DREB1B-downstream genes instead of the DREB1A-downstream genes.

It has been reported that ICE1 functions only in the regulation of *DREB1A* expression and that CAMTAs function in the regulation of both *DREB1B* and *DREB1C* expression, indicating that these proteins function independently.

Finally, please place more emphasis on novel aspects of the paper rather than on what is an extension of previously reported findings.

RESPONSE: To emphasize the novel aspects of the paper, we revised the manuscript, especially the Discussion section (P. 19, line 376-400).

Reviewer #1:

In this work the authors assess the contribution of the CAMTA family of proteins to cold-induced gene expression. The authors document the tissue specificity, subcellular localization and induction of transcript accumulation in response to cold stress before proceeding to define DNA binding motifs. The authors use high-order mutants to examine the function of individual *CAMTA* genes- *CAMTA3* and *CAMTA5* are the predominant family members. Subsequent analysis suggests that while cold-induced signalling occurs independently of CAMTAs (although a role for residual CAMTA2 cannot be excluded), some CAMTA-specific gene induction is also observed.

Overall, the creation of a sextuple *camta123456* mutant (and related quintuple mutants) certainly provides a valuable resource for future work. However, attempts to link observed data with circadian control would benefit from additional work.

1) Line 123- "two different signals" I don't think evidence is provided in this manuscript that suggests that cold sensitivity occurs via two different signals- the authors should rephrase.

RESPONSE: We rephrased this text (P. 5, line 123) as follows: "plants respond differently to rapid and gradual temperature decreases."

2) Line 133- It would be useful to present the data from the Y1H screen that identified CAMTA2.

RESPONSE: We included the data from the yeast one-hybrid screen in Supplemental Table 1 and also presented these data in the Results section (P. 6, line 136).

3) In the abstract, the authors state that *CAMTA3/5* are induced in response to a rapid decrease in temperature, but these data are not presented (only *CAMTA4* is induced in Figure 1). Similarly- line 32- "these proteins are not activated"- but the authors do not examine 'protein activation'- please clarify.

RESPONSE: We revised this sentence in the abstract (P. 2, lines 31-33) as follows: "Calmodulin-binding transcription activator 3 (CAMTA3) and CAMTA5 respond to a rapid temperature decrease and induce the expression of *DREB1s,* but these proteins do not respond to a gradual temperature decrease."

4) The authors should include an image of DAPI-stained nuclei (or other appropriate stain) to confirm that CAMTA2-, CAMTA3- and CAMTA5-GFP are localized to the nucleus (Figure 1C). Normalization of *CAMTA* expression levels in Figure 1A is also unusual- why not just show data relative to a reference gene?

RESPONSE: We added an image of DAPI-stained nuclei as Supplemental Figure 2 to demonstrate that these CAMTA proteins are localized to the nucleus (P. 7, lines 158-162; P. 25, lines 554-555). According to this comment, expression of the *CAMTA* genes is presented relative to that of the reference gene in Figure 1, as explained in the legend of this figure.

5) As SA signaling is presumed to be unregulated in all of the *camta* higher order mutants (except *camta12456*) could the authors examine SA-responsive genes using qPCR (in addition to the biosynthesis genes described) to confirm whether SA signaling is correlated with the dwarf phenotype?

RESPONSE: We analyzed the expression of *PR1*, an SA-induced gene, and *XTH31,* an SA-repressed gene, using qPCR, and added the results to Figure 3D and explained these data in the Results section (P. 11, line 230 - P. 12, line 236; P. 13, line 292 - P. 14, line 296).

6) In Figure 4B, is this qRT-PCR or data extracted from RNAseq? If the latter, more stringent statistical methods should be used rather than a *t*-test.

RESPONSE: This figure shows the qRT-PCR results. We changed Figure 4 to Supplemental Figure 6 in the revised manuscript according to reviewers' suggestion. In addition, we added a description of the statistical methods to the legend of Supplemental Figure 6.

7) Line 233. It is unclear to me why the authors compared genes down regulated in *camta123456* mutants with genes unregulated in *DREB1Aox*. Figure 3B shows that *DREB1A* expression is only modestly down-regulated in

camta123456 compared to WT, so why is this comparison informative? The authors should also clarify in the text if the transcripts assessed in Figure 4B are also downstream of DREB1B and DREB1C.

RESPONSE: Because DREB1A/CBF3, DREB1B/CBF1 and DREB1C/CBF2 have been shown to regulate similar sets of cold-inducible genes (Park et al., 2015), we compared the down-regulated genes in *camta123456* with the up-regulated genes in DREB1A ox in our previous manuscript. According to this comment, we compared the down-regulated genes with the up-regulated genes in *DREB1B-ox* and obtained similar results as those previously reported. We presented these new results instead of the old results in Supplemental Figure 6 and explained these data in the Results section (P. 12, lines 246- 257).

8) Line 279- "CAMTA proteins did not function in the plants grown on agar plates"- but the authors have not presented data examining whether *CAMTA* genes are expressed when plants were grown on agar. This could be an alternate explanation.

RESPONSE: We analyzed expression of the *CAMTA* genes in plants grown on agar plats and in soil pots according to this comment. The results showed that *CAMTA* gene expression was similar between the two groups of plants. We included these results in Supplemental Figure 7 and explained these data in the Results section (P. 13, lines 279-281).

9) Line 307- Could the authors please clarify; were these experiments performed sequentially or simultaneously? I.e. were plants stepped down from 22-19-16-etc, or were they transferred immediately from 22°C to the indicated temperature? If the former, how can the authors exclude circadian/diurnal effects?

RESPONSE: Plants were transferred immediately from 22°C to the indicated temperature. We added this information to the Results section (P. 15, lines 328-331) and to the legend of Supplemental Figure 10.

10) Line 314- Could the authors please clarify their treatment regime? Were plants treated on day '0' and then transferred into constant light? Or were plants kept in entraining conditions? The authors mention 'subjective night' but describe plants as being grown in light:dark cycles. Were different plants treated and harvested at each time point? This is not clear from the figure legend, and a description is lacking in the methods section. These alternatives significantly alter my interpretation of the data.

RESPONSE: We newly analyzed the effects of circadian rhythm on cold-inducible expression of the *DREB1* genes and included the results in Supplemental Figure 11 instead of the previous Supplemental Figure 6. In addition, we precisely described the experimental procedure in the Results section (P. 17, lines 337–341) and in the legend of Supplemental Figure 11.

11) Line 334- "in contrast to previously study (Dong et al 2011)". The authors state *DREB1B* is synonymous with *CBF1* (Introduction). Their data shows that *DREB1B/CBF1* expression in WT is similar to that in *cca1 lhy* (Figure 6C). Dong et al show that *DREB1B/CBF1* induction is gated by the clock; this result is not reproduced in this study. However, the authors' methodology needs to be described in more detail before these two datasets can be compared (see also Comment 10).

RESPONSE: We also newly analyzed the effects of circadian rhythm on cold-inducible expression of the *DREB1* genes in *camta35* and *cca/lhy* mutants and included the results in Figure 6. In addition, we precisely described the experimental procedure in the Results section (P. 17, lines 348-352) and in the legend of Figure 6.

12) Figure 6C- CAMTA3 has previously been reported to positively regulate *DREB1B* (Doherty et al 2009)- the opposite of the result here. Can the authors suggest why their results are so different?

RESPONSE: *DREB1B* expression was clearly reduced in the *camta35*, *camta3* and *camta5* mutants (Figures 4B and Supplemental Figure 8 in the revised manuscript). Therefore, we think that CAMTA3 and CAMTA5 positively regulate *DREB1B/CBF1*. These results are similar to those of a previous CAMTA3 report (Doherty et al 2009).

13) Figure 7- It's a bit atypical to have the clock 'absent' in a schematic at different times- the authors should re-draw, either with 'clock' but without the arrows, or with another method to illustrate the proposed interaction

RESPONSE: We revised Figure 7 in accordance with this helpful comment.

14) Line 390 "CAMTA2/3 mainly suppress SA biosynthesis" Why have the authors both CAMTA2 and CAMTA3 here? Only the *camta12456* quintuple appears to have a significant difference in *EDS1* or *ICS1* expression.

RESPONSE: According to this comment, we revised this sentence (P. 21, lines 427-429) as follows: "In this study, we showed that CAMTA3 plays an important role and that CAMTA2 has a minor role in the suppression of SA biosynthesis using *CAMTA* quintuple mutants (Figure 3)."

15) Line 407- the authors do not demonstrate that SA is synthesized in the sextuple mutant

RESPONSE: According to this comment, we revised this sentence (P. 22, line 447) as follows: "SA was likely synthesized in the sextuple mutant that lacked these *CAMTA* genes,"

16) Supplemental Figure 4- The authors should include temperature data for open/closed agar plates to aid interpretation of this figure.

RESPONSE: According to this comment, we added the temperature data for the open/closed agar plates to Supplemental Figure 9A in the revised manuscript.

17) The utility of Supplemental Figure 6 is limited, as the authors have chosen to assess transcript accumulation at ZT2 and ZT14. This means it is not possible to separate light-induced gene expression (following dawn- ZT0) from circadian effects. It would be more usual to assess circadian effects after at least 1 day in free-running conditions (i.e. ZT26 and ZT38).

RESPONSE: According to this comment, we newly analyzed the effects of circadian rhythm on cold-inducible expression of the *DREB1* genes and presented the results as Supplemental Figure 11 instead of the previous Supplemental Figure 6. In this experiment, we assessed circadian effects after 1 day under free-running conditions. We precisely described the experimental procedure in the legend of Supplemental Figure 11.

18) Figure 4- I was surprised that COR15A accumulation was not significantly down-regulated at time points 6 and 12 compared to WT- could the authors please confirm?

RESPONSE: In response to this helpful comment, we examined these data and found that *COR15A* expression was significantly down-regulated in *camta123456* at the 6 h and 12 h time points compared with that in the WT. We included the results of statistical analysis of the qRT-PCR data in the Supplemental Figure 6B.

19) Does Supplemental Figure 3B indicate accumulation of full-length transcript? The use of RT-PCR to confirm loss of indicated transcripts may be appropriate in this context, but the methodology used is not described in the Methods section.

RESPONSE: We described the RT-PCR methods in the Methods section (P. 25, lines 539-540) as suggested.

Reviewer #2:

In this manuscript by Kidokoro, Yoneda, and colleagues investigate the role of CAMTA transcription factors in the regulation of DREB1s. CAMTAs have previously been shown to have a role in low temperature regulation of *DREB1s*, but this manuscript provides more detail into the transcriptional regulation of the *DREB1* genes in response to low temperature. The experiments and analysis performed here provide novel insights into how plants sense temperature and the difference in the molecular response to rapid versus gradual temperature decreases. This is a well-written manuscript that contributes to the understanding of the low temperature regulatory network. Clarification is needed in the some aspects of the experimental analysis and there are some modifications that would improve the overall manuscript.

In all figures, please indicate how "significantly different" is assessed in all comparisons. For example, the authors indicate that *CAMTA4* is transcriptionally induced in response to cold treatment but the other 5 *CAMTAs* are not. How do the authors discern that there is not a significant difference for *CAMTAs* 3 & 5? Also for Figure 3C, the authors indicate repression of *DREB1B* and *DREB1C*, but not *DREB1A*. How is this determination made? Three of the

mutant combinations appear to have an effect that is significant in reducing DREB1A levels. This is also needed for clarification of the results in Figure 5B, Supplemental Figure 5, 6B, Supplemental Figure 6, and especially 6C.

RESPONSE: We statistically analyzed all of the data indicated in this comment (Figures 1A, 3C and 3D, 4B and 4C, 5B, and 6 and Supplemental Figures 8-11 in the revised manuscript). We indicated how "significant differences" were assessed in all comparisons in the legends of these figures.

Related to the point of significance, how are the qRT-PCR replicates incorporated? Specifically what type(s) of replicates are used here for the error bars? P24, line 495. qRT-PCR. "Triplicate measurements were performed for each cDNA sample" it is not clear how many samples were performed for each genotype/treatment. Were three RNA samples prepared for each treatment (3 WT warm, 3 WT cold-stress, 3 *camta1,2,3,4,5,6* cold-stressed) and then 3 measurements on each sample. If so, how were the errors combined? Or was one sample collected with three measurements performed and this is what is included in the figure? It is indicated in the figures that the error bars indicate the standard deviation, is this of the technical replicates, the biological replicates, or the combination of the two?

RESPONSE: We explained our qRT-PCR method in the Methods section (P. 25, lines 541-548) and in the figure legends.

Please indicate in materials and methods, what time of day these experiments were performed. Given the variability observed with time of day, this is important information for anyone interested in the regulation of *DREB1s*.

RESPONSE: We indicated the time of day at which these experiments were performed in the figure legends.

P 6, Line 140 and Supplemental Figure 1. Results concerning the phylogenetic analysis: A cross-species phylogenetic analysis of the plant *CAMTA* genes has previously been performed (Rahman *et al*. *Frontiers in Plant Science* 2016) and is not cited by this manuscript. This previous works comes to a different conclusion about the origin of the plant *CAMTAs* and this discrepancy should be addressed, perhaps in the discussion.

RESPONSE: We revised the following sentence and cited this paper (Rahman et al. 2016) in the Results section (P. 6, lines 146-148): "*Physcomitrella patens* has only the *CAMTA1/2/3* subtype genes, indicating that all six *CAMTA* genes are likely derived from common ancestors, as recently reported (Rahman *et al*., 2016)"

P7, line 157. The subcellular localization for CAMTA2, CAMTA3, and CAMTA5. Nuclear localization of CAMTA3 and CAMTA5 proteins (portion of Fig 1C) was previously published in 2002, Yang and Poovaiah, JBC. (CAMTA3); 2003, Mitsuda et al. *Plant Cell Phys* (CAMTA5) and should be referenced. The wording of this section does not make it clear if all CAMTAs were tested for nuclear localization or only these three. It appears from the methods section that they were not tested. This should be indicated in the main text. "Subcellular localization was tested in CAMTA2, CAMTA3, and CAMTA5 ..." Otherwise it leaves open questions to the readers- Were they tested and not observed to be nuclear localized or were they not tested? Either way it is fine, but as written, it implies they were tested and are not nuclear localized, which doesn't appear to be the case based on the description in the methods section.

RESPONSE: According to this comment, we revised this section and cited these two papers (P. 6, line 155 - P. 8, line 164) as follows: "The nuclear localization of GFP-fused CAMTA3 and CAMTA5 driven by the CaMV *35S* promoter has been previously demonstrated using transient transformation (Yang and Poovaiah, 2002; Mitsuda *et al*., 2003). We analyzed the subcellular localization of the 6 CAMTA proteins fused to synthetic GFP (sGFP) (Chiu et al., 1996) and driven by their own promoters and the CaMV *35S* promoter in transgenic plants. The sGFP fusion proteins of CAMTA2, CAMTA3 and CAMTA5 were found to be localized to the nuclei of the Arabidopsis plants at room temperature (Figure 1C; Supplemental Figure 2). However, GFP fluorescence of the fusion proteins of CAMTA1, CAMTA4 and CAMTA6 was not observed at room temperature or at 4°C."

We also revised the sentence in the Methods section to increase clarity (P. 24, lines 525-526).

P11, line 223. "These findings indicate that CAMTA2 and CAMTA3 mainly function in the suppression of SA biosynthesis." As written it sounds like this is the primary role for CAMTA2 and CAMTA3 (which contradicts the previous paragraph for the role of CAMTA3 in cold-responsive expression of DREB1B and DREB1C) rather than

CAMTA2 and CAMTA3 are the primary CAMTAs involved in SA biosynthesis, which is probably the authors' intended meaning.

RESPONSE: According to this comment, we revised this sentence (P. 12, lines 236-238) as follows: "All of these findings indicate that CAMTA3 plays a major role and that CAMTA2 has a minor role in the suppression of SA biosynthesis."

P11, line 226. RNA-Seq- it would be interesting to see the results of the DREB1s in the RNA seq analysis, to confirm the results observed in figure 5. This would be sufficient as a supplemental figure.

RESPONSE: The RNA-seq results revealed that *DREB1B* and *DREB1C* were down-regulated in the *camta* mutant but that *DREB1A* was not down-regulated, which confirmed the results presented in Figure 4. According to this comment, we changed Figure 4 to Supplemental Figure 6 in the revised manuscript.

Figure 6, please indicate on the panel in figure A what time the 1h samples were harvested in the Rapid and Slow response- was it at the same 60 min mark in both, or at 60 in the rapid and 110 min (60 min + 50 min, when the plants reached 4C)?

Also Figure 6 and Supplemental Figure 6. How were these analyzed on the qRT-PCR if the 96 well instruments indicated was used? How were they broken up - were the technical replicates run on separate plates? The time points? So, for example, in Supplemental Figure 6, were the ZT2 and ZT14 samples run in the same plate or were they done sequentially?

RESPONSE: We included the time points in the legend of this figure (Figure 5A in the revised manuscript). We also revised the legend and Methods section (P. 24, lines 510-513) to clearly explain both the rapid and slow cold treatments. In addition, the qRT-PCR method is described in the Methods section (P. 25, lines 541-548), as well as in the legends of the new Figures 5 and 6 and Supplemental Figure 11.

P21, line 431. " As plants grown on agar plates were use, only the effect of the slow temperature decrease could be assessed in the previous study." It is impossible for the authors to know if the temperature decrease on plates in the previous study was fast or slow. There are likely to be numerous differences between the previous study and the experiments performed in this manuscript (light intensity, growth temperature, density of planting, photoperiod- to name a few). It is not appropriate to assume the difference is due to the temperature decrease. Simply saying there is a difference in the observed results is sufficient.

RESPONSE: According to this comment, we revised this sentence (P. 22, lines 470-472) as follows: "These discrepancies between the results of the previous study and our findings might be attributed to differences in experimental conditions."

P 25, line 539. Please ensure that the raw RNA-seq reads are available on NCBI, they are currently no results for the indicated accession number on NCBI.

RESPONSE: We have uploaded the raw RNA-seq reads to NCBI. They will be available after acceptance of this manuscript.

Reviewer #3:

Arabidopsis DREB1/CBFs are critical regulators of plant responses to cold/frozen stresses and their induced expression are usually correlated with increased tolerance to cold/frozen stresses. Among the regulatory factors involved in the expressional regulation of *DREB1s*, CAMTAs are believe to provide mechanisms coupling the cold triggered Ca^{2+} transients to the cold/frozen responding signaling network. In the current manuscript, the authors reported their studies of transcriptional control of three Arabidopsis DRE-binding protein 1/C-repeat binding factors (*DREB1/CBFs*) by calmodulin-regulated CAMTAs. Their results showed that plants are able to discriminate between cold stresses applied in two different patterns, namely the rapid and gradual temperature decreases. While *DREB1A* was found to be induced by both rapid and gradual temperature decreases, *DREB1B* and *C* were shown to be drastically induced only by rapid decrease in temperature, and Arabidopsis CAMTA3 and CAMTA5 were found to be

involved in this process. Furthermore, CAMTA3 and CAMTA5 mediated induction of *DREB1B* were found to be mostly independent of circadian rhythm while the induction of *DREB1A* by cold treatments is affected.

Overall, the discoveries, especially the role of CAMAT3 and 5 as regulators of *DREB1B* and *C*, the ability of plants to recognize different patterns in cold treatments, are novel and should be of great interesting to plant scientists working in related areas such as Ca^{2+} signaling and stress responses. I would like to see the paper get published after addressing a few minor issues.

1. Stress-triggered changes in gene expression, as well as spatial pattern of the gene products are generally believed to correlate with their functional significance. The authors studied the cold induced expression of all the 6 *CAMTA* genes, and the role of cold-responsive *CAMTA4* in regulating cold response is not found, instead, the non-responsive *CAMTA3* and *5* were shown to play critical roles in regulating cold responses. This discrepancy should be discussed somewhere in the result section or discussion section.

RESPONSE: According to this comment, we added the following text to the Results section (P.21, lines 434-440): "As expression of the *CAMTA3* and *CAMTA5* genes are not induced by cold stress, it is important to analyze their activation mechanisms in response to cold stress. Requirement of Ca^{2+} signaling for the regulation of CAMTA activity has not been observed in the cold stress response, but because the calmodulin-binding site is also conserved in CAMTA5 and involvement of Ca^{2+} signaling has been reported in the cold stress response, Ca^{2+} signaling may also be important for the regulation of cold-responsive CAMTA activity (Townley and Knight 2002; Liu et al., 2015)."

2. In the transactivation assays using promoter::ELUC combined with different CAMTA effectors, the authors found that CAMTA3 and CAMTA5 are the two CAMTA members regulating the transcription of *DREB1B* and *DREB1C* (Figure 1B and Supplemental Figure 2B) with CAMTA3 works better than CAMTA5. However, they chose to test only the binding of CAMTA5 to the *cis*-elements in the promoter of *DREB1s*. I think both CAMTA3 and CAMTA5 should be tested for their interaction with these *cis*-elements, or the authors should give appropriate justification for their biased selection.

RESPONSE: We performed EMSA using the CAMTA3BD protein and found that this protein bound to the promoter regions of *DREB1B* and *DREB1C,* similar to CAMTA5BD. We included these results in Supplemental Figure 4 and explained these data in the Results section (P. 9, line 198 - P. 10, line 199).

3. As mention above, in the transactivation assay, authors found that CAMTA3 could work better than CAMTA5 in activating *DREB1B* and *C*. While examining the expression of DREB1B and DREB1C in quintuple mutants, their results indicated that CAMTA5 are more effective in mediating the cold induced expression of these DREB1 genes. Appropriate explanation should be provided to address the potential reason for these differences.

RESPONSE: Using single *camta* mutants, we found that CAMTA3 was a stronger activator of *DREB1B* and *DREB1C* than CAMTA5 (Supplemental Figure 8). Interactions among CAMTA proteins might affect the activity of these proteins. Further analysis is necessary to clarify the activation mechanisms of CAMTA proteins.

4. The contribution of transcriptome analysis to the main conclusion of the research is not adequately presented.

RESPONSE: According to the reviewers' suggestion, we moved the RNA-seq data from Figure 5 to Supplemental Figure 6. We also compared the down-regulated genes in the *camta* mutants with the cold-inducible genes and DREB1B-downstream genes instead of the DREB1A-downstream genes.

5. The discrepancy in phenotypes of WT, *camta23*, *camta35*, and *camta123456* plants grown in soil pots and on agar plates leads the authors to discover that the rapid and gradual temperature decreases are different in inducing the expression of *DREB1* genes (figure 5). In wild-type plants, rapid temperature drop was shown to induce humongous transcriptional elevation of *DREB1A*, *B*, and *C* and gradual temperature decrease was shown to cause similar induced-expressional jump in *DREB1A* expression but not in the induction in *DREB1B* and *C*, indicating that a rapid temperature drop is an effective treatment to study the transcriptional control of *DREB1B* and *C*. Furthermore the induction of *DREB1B* and *C* by rapid temperature drop was shown to be affected by *CAMTA* genes, particularly *CAMTA3* and *5*. Considering the confused individual roles/contribution of CAMTA3 and 5 as mentioned above,

adding single mutants of *camta3* and *camta5*, as well as mutants other than in these two genes (as a kind of reference, such as *cam14* or *cam1246*) in the comparative studies presented in Figure 5 (and also in Fig. 6, Suppl. Fig.4) will further strengthen this manuscript.

RESPONSE: In response to these suggestions, we conducted these experiments corresponding to Figures 5 and 6 in the previous manuscript using *camta3, camta5*, and *camta4* single mutants. We added the results to Supplemental Figure 8 and explained these data in the Results section (P. 15, lines 315-319).

TPC2016-00669-RAR1 2nd Editorial decision – *accept with minor revision* Feb. 2, 2017

The reviewers collectively agree that the manuscript can prove suitable for publication in *The Plant Cell*, has novel and important findings, and that you have substantially addressed their prior concerns. However, there were still some critical issues that must be fixed prior to acceptance and some minor issues that should addressed.

[Editor comments shown below with author responses.]

TPC2016-00669-RAR2 2nd Revision received Feb. 19, 2017

Editor and reviewer comments and **author responses**:

Editor:

1. qPCR statistics needs to be undertaken and presented on the biological replicates, not technical replicates, with representative images shown. This has also been raised earlier *Plant Cell* articles (Udvardi 2008) and another article on presentation of qPCR data and its analysis is Bustin, S.A. *et al. Clinical Chemistry* 55:4 may be of use to you. As you indicate in the methods that the data is available this should be straightforward.

Note that the sampling and nature of "biological replicates" should be described precisely (*i.e.* different plants, parts of plants, pooled tissue, independent pools of tissue, sampled at different times, etc). The reader should know exactly what was sampled; what forms the basis of the calculation of any means and statistical parameters reported. This is also necessary to ensure that proper statistical analysis was conducted.

RESPONSE: All qRT-PCR experiments in this manuscript were performed three times using the plants sampled at different times. Average and standard deviation of the three biological replicates were shown in the figures. The significance of expression changes was evaluated according to the expression levels of the genes calculated from the biological replicates. In response to this comment, we provided a similar explanation not only in the Methods section but also in each figure legend.

2. Do the correct statistical test for the point you want to make. There are places where the point is to compare one thing (e.g. agar vs soil) but the test they do is for another comparison (mutant vs wt).

RESPONSE: In response to this comment, we have performed statistical tests of the expression levels of the genes in the plants under all treatment conditions, and the results of these tests are presented in the new Figure 4B, 4C and Supplemental Figure 8.

3. Adjust claims in the manuscript if some findings are no longer significant after the forementioned analyses are undertaken.

RESPONSE: All our findings were significant after the analyses were performed.

4. Address minor and major comments as much as practical in the manuscript and in a response to reviewers' letter

RESPONSE: We have addressed all minor and major comments in the responses to the reviewer's comments and revised our manuscript according to their reasonable suggestions.

5. The physiological data suggested by Reviewer #3 is not required, but can be provided if available.

RESPONSE: We have not analysed the freezing tolerance of the *camta5* and *camta35* mutant plants. We would like to obtain these data suggested by Reviewer #3 in the future.

Reviewer #1:

The authors have significantly improved their manuscript with the additional data provided. I have the following comments regarding their revised manuscript.

Line 163- If CAMTA 1,4, 6 aren't in the nucleus where are they? Was this a consequence of reduced protein accumulation in these lines? Or were these tagged proteins present in another subcellular compartment?

RESPONSE: We have revised this sentence as shown below in the response to this comment. "However, no GFP fluorescence of the fusion proteins of CAMTA1, CAMTA4 and CAMTA6 was observed at room temperature or at 4°C in any part of the cell. These CAMTA proteins might be unstable under these conditions." (P. 7, line 157 - P. 8, line 159)

Line 180- expression of CAMTA3+5 is not similar between soil and agar- they are twice as high on agar. These data address my initial concern (that CAMTAs were not expressed in agar-grown plants), but the current wording is inaccurate.

RESPONSE: We have revised this sentence as shown below. "We examined the expression of the 6 *CAMTA* genes in WT plants and found that these genes were expressed in both plants grown in soil pots and plants grown on agar plates (Supplemental Figure 7)." (P. 13, lines 274-277)

Line 240- the authors should work in Zeitgeber time rather than Circadian time. Zeitgeber time= time since last environmental signal. Circadian time= calculated endogenous time, which varies depending on the circadian rhythm of the cell- circadian time is reset after each oscillation (e.g. ZT30 is CT6, assuming the cell has a 24-hr circadian period).

RESPONSE: We have revised this description throughout the manuscript according to this suggestion.

Line 349: "was assessed...after the diurnal cycle"? Perhaps this could be re-written to "was assessed at several time points following transfer to free-running conditions."

RESPONSE: We have revised this sentence as the reviewer suggested. "Further, cold-inducible *DREB1* expression was assessed at several time points in *camta35* and WT (Col-0) plants following transfer to free-running conditions (Figure 6)." (P. 17, lines 346-347)

Reviewer #2:

This revision addresses some of the major concerns. However, not all concerns have been fully addressed. Overall the first portion of this manuscript concerning the role of the CAMTAs in the regulation of *DREB1A*, *1B*, and *1C* is clear and well presented. The findings about the difference in response based on the rate of temperature drop is very exciting and will be informative to the study of low temperature response networks. However, the last portion of the manuscript, on the distinction of the response between CAMTAs and the circadian clock is not very convincing to this reviewer and the interpretations do not seem well supported by the presented data. Of primary concern throughout the manuscript is the method of analysis of qRT-PCR results. Determining significance from technical error is not very robust and can lead to misinterpretation and flawed claims, especially when the results are a less than two fold difference, which would be less than one CT value.

RESPONSE: All experiments for qRT-PCR were performed three times, using the plants sampled at different times. In response to this comment, the significance of expression changes was evaluated according to the expression levels of the genes calculated from the biological replicates.

The circadian section would be enhanced by more support for the claim that *DREB1B* and *DREB1C* are not circadian regulated. The materials and methods now clearly describe the experiments and analyses performed. Detailed comments are below.

RESPONSE: We think that the regulation of *DREB1* gene expression by the circadian clock is not so simple. We revised the Discussion section as shown below to explain the circadian regulation of the three *DREB1* genes under cold stress.

"*DREB1A* and *DREB1C* contain an evening element (EE; AAATATCT), which is a CCA1/LHY binding site, near the TATA boxes in their promoters, and *DREB1B* contains an EE at approximately 350 bp upstream of the TATA box in its promoter. Therefore, it seems that *DREB1A* and *DREB1C* are strongly and *DREB1B* is weakly regulated by CCA1/LHY. We detected their certain involvement in the cold-responsive expression of *DREB1A* and *DREB1C* using *cca1/lhy* (Figure 6). However, these genes were still expressed in *cca1/lhy,* although their expression was significantly reduced. Moreover, the cold-responsive expression of *DREB1B* was not clearly altered in *cca1/lhy,* in contrast to a previous study (Dong et al., 2011) that showed that the expression of *DREB1A/CBF3*, *DREB1B/CBF1* and *DREB1C/CBF2* is nearly absent in *cca1/lhy*." (P. 22, lines 462-472)

"One signal is caused by a rapid temperature drop to less than 10°C. In this signaling pathway, CAMTA3 and CAMTA5 mainly function as transcriptional activators in the expression of *DREB1B* and *DREB1C*. The other signal is caused by both rapid and gradual temperature decreases to less than 10°C. The central oscillator of the circadian clock, involving CCA1/LHY, may play a role in this signaling pathway to strongly induce the expression of *DREB1A* and *DREB1C* and weakly induce the expression of *DREB1B*." (P. 23, lines 483-489)

Line 296: Thus, *EDS1*, *ICS1*, and *PR1* were repressed and *XTH31* was expressed in the plants grown on agar plates, despite the absence of *CAMTA* gene expression. These are not repressed (expressed *XTH31*) in the *camta123456* mutant as this statement implies. These genes are low in the WT, which has *CAMTA* so the absence of *CAMTA* is not the variable that is associated with the change in expression.

RESPONSE: We have revised this sentence as shown below. "Thus, *EDS1*, *ICS1* and *PR1* were repressed and *XTH31* was expressed in the plants grown on agar plates, regardless of the presence of the *CAMTA* genes." (P.14, lines 291-292)

Line 304: The title of this section indicates that there is comparison between day and night, but there is no comparison between day and night here.

RESPONSE: In response to this comment, we revised the title as shown below. "CAMTA proteins function in cold-inducible expression of *DREB1B* in response to rapid temperature decrease." (P.15, lines 300-301)

Figure S10- reduction in DREB1A levels at 4C after 1h in the camta123456 mutant appears inconsistent with data from previous figures showing no effect of the sextuplet mutant on DREB1A expression.

RESPONSE: In Figures 3 and 4, plants were treated for 3 hours at 4°C but in Figure S10, they were treated for 1 hour. Since the *camta123456* mutant plants grown in soil pots exhibited growth retardation and early leaf senescence, there might be indirect effects of the growth inhibition on the induction of *DREB1A* expression at 1 hour. As shown in Figure S10, the expression of *DREB1A* was similarly induced in the *camta123456* mutant and WT, similar to the data in Figures 3 and 4. Three hours appears to be sufficient to increase the expression of *DREB1A*, even in the *camta123456* mutant plants that exhibit growth inhibition.

Figure 5: The caption of this figure indicates that there is a comparison of the induction in diurnal cycles, but no such comparison is made.

RESPONSE: In response to this comment, we removed "under diurnal cycles" from the caption of Figure 5.

Line 343/ Figure S11: The point of this figure based on the text is the comparison between treatment in the morning and treatment at night. However, either there is no significant difference between the points or this was not evaluated. *DREB1B* Expression is also lower in treatment at night, or the induction rate has changed. For example at 3 h after treatment, the level of *DREB1B* in the PM is half what it was in the AM, this difference was considered meaningful in other figures. *DREB1C* appears to look exactly like *DREB1B* in terms of both sensitivity to time in the

circadian cycle and response to the *camta* mutant background. Please indicate how this exhibits combined characteristics of *DREB1A* and *DREB1B*.

RESPONSE: In response to this comment, we have revised the sentence as shown below. "*DREB1C* expression exhibited characteristics of both *DREB1A* and *DREB1B* expression, with reduced expression in *camta35* and *camta123456* in both subjective day and night compared with that in the WT plants and lower levels of expression in the subjective night compared with those in the subjective day." (P. 17, lines 342-345)

Figure 6/ S11B. These qRT-PCR results are normalized to WT warm levels (set to 1), but what are those levels in the Ws background? This is of particular importance for *DREB1B*, where the induction is much lower than what would be expected in wild-type plants. It is concerning because if there is an issue with the measurement not being at the typically high response of 1B (primers not working in Ws / measuring a different time in the kinetics of induction), then the fact that a reduction is not observed in the *cca1/lhy* mutant is not informative. Why is *DREB1C* not included in this picture? This would simply be one additional qRT-PCR analysis. Again these two figures clearly support the role of CAMTAs in specifically regulating *DREB1B* and not *DREB1A*. However, the results from the circadian analysis, are minimal- only one mutant line is evaluated, and there, the level of *DREB1B* in wild type plants is surprisingly low after cold induction.

RESPONSE: As the reviewer indicated, the induction of *DREB1B* in WT (Ws) was much lower than expected. Therefore, we analysed *DREB1B* expression in WT (Ws) and *cca1/lhy* using the different, newly synthesized primers. In this analysis, the expression levels of *DREB1B* in WT (Ws) were not considerably different from those in WT (Col-0). We changed these data in Figure 6 and Figure S11. We also analysed the expression of *DREB1C* using *camta35* and *cca1/lhy*, and the data are included in Figure 6 according to the reviewer's suggestion. We revised the Results section concerning these data in the manuscript as shown below.

"*DREB1C* was expressed in a circadian manner, but its expression was higher than that of *DREB1A* during the subjective night. Moreover, *DREB1C* expression was significantly decreased in *camta35* at all time points. As the cold-inducible expression of *DREB1s* has been reported to be reduced in *cca1/lhy* double mutant plants (Dong et al., 2011), we further analyzed the expression of *DREB1s* in *cca1/lhy* (Figure 6). The cold-inducible expression of *DREB1A* and *DREB1C* at ZT6, ZT10 and ZT30 was lower in the mutant than in the WT (Ws), but obvious expression of both genes was still detected in the mutant, suggesting that other factors are also involved in their expression. However, *DREB1B* expression was not significantly altered in *cca1/lhy*, in contrast to the findings of a previous study (Dong et al., 2011)." (P. 17, line 356 - P. 18, line 365)

Data are not available on PRJNA326982- on GEO, a search for that ID returns nothing.

RESPONSE: The data have been released and are available on PRJNA326982.

Figure 3. B. The letters above the bars should have an appropriate comparison key to help the reader understand their significance. It is not standard to have a "bc" comparison without a "c". Why are these not "b"?

RESPONSE: In response to this comment, we revised the letters above the bars in Figure 3B.

Figure 3 C. The figure legend says that the values are relative to WT level after cold treatment (set to 100). However, for *DREB1B*, WT level after cold appears to be set to 80, even including the error. The increase in *DREB1B* and *DREB1C* in the *camta12346* mutant is questionable as this is less than 2-fold difference, which would be less than one CT value and only technical replicates (and not biological variation) is included in the statistical analysis.

RESPONSE: We have corrected the error in Figure 3C. We performed all qRT-PCR experiments in this manuscript three times, using the plants sampled at different times, and determined significance from biological replicates. As shown in Figure 3C, we also performed biological replicates, and significant increases in *DREB1B* and *DREB1C* expression in *camta12346* were detected. We have explained the biological replicates not only in the Methods section but also in each figure legend to avoid a misunderstanding about our method of qRT-PCR.

Line 280 / Figure S7. Comparison between *CAMTA* levels in soil and agar grown plants remains similar. Was this comparison (which seems to be the point of the figure) tested for significance? The fold change between soil and agar for *CAMTA3* and *5* is as large as those in the other figures that were identified as significant, with small error

bars? Was the expression level the same in response to low temperature (since this is really when the effect would matter)?

RESPONSE: In response to this comment, we performed statistical tests of the expression levels of *CAMTA* genes in plants grown in soil and on agar, and the results of these tests are shown in Figure S7. We also revised the sentence on previous line 280 as shown below. "We examined the expression of the 6 *CAMTA* genes in WT plants and found that these genes were expressed in both plants grown in soil pots and plants grown on agar plates (Supplemental Figure 7)." (P. 13, lines 274-277)

Reviewer #3:

In the revised manuscript, the authors addressed the most important issues I raised regarding the previous version: 1) they tested the binding of CAMTA3 to the *cis*-elements in the promoter of *DREB1s*, and confirmed similar interaction patterns as those interactions between CAMTA5 and the cis-elements in *DREB1s'* promoters (issue #2); 2) they examined the expression of *DREB1A* and *DREB1B* in single mutant plants of *camta3*, *camta5*, and *camta4* (served as an reference) grown in soil treated with quick and slow decrease in temperature and in sealed agar plates treated with quick temperature decrease, and their results confirmed that CAMTA3 and CAMTA5 play additive roles in the induction of *DREB1B* by cold shock (issue #5). Furthermore the use of single mutants indicates that CAMTA3 is more effective in mediating the induction of *DREB1* genes by quick temperature drop, helping clarify a minor concern (issue #3). The other two concerns are relatively very minor, and many readers could easily understand.

Considering this is the first time to report that CAMTA5 plays a role in cold response, there is still a weakness in lacking of physiological data to show that *camta5* single mutant and *camta3 camta5* double mutants are compromised in cold or freezing tolerance.

RESPONSE: We would like to examine the freezing tolerance of the *camta5* and *camta35* mutant plants in our future study.

TPC2016-00669-RAR2 3rd Editorial decision – *acceptance pending* **Mar. 9, 2017**

We are pleased to inform you that your paper entitled "Differential signaling in cold responses to rapid and gradual temperature decreases in Arabidopsis" has been accepted for publication in *The Plant Cell*, pending a final minor editorial review by journal staff.

Final acceptance from Science Editor **Mar. 22, 2017**