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Ca²⁺-permeable mechanosensitive channels MCA1 and MCA2 mediate cold-induced cytosolic Ca²⁺ increase and cold tolerance in Arabidopsis

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Cold shock triggers an immediate rise in the cytosolic free calcium concentration ($[Ca^{2+}]_{cyt}$) in *Arabidopsis thaliana* and this cold-induced elevation of $[Ca^{2+}]_{cyt}$ is inhibited by lanthanum or EGTA. It is suggested that intracellular calcium mainly contributes to the cold-induced $[Ca^{2+}]_{cyt}$ response by entering into the cytosol. Two calcium-permeable mechanosensitive channels, MCA1 and MCA2 (*mid1*-complementing activity), have been identified in Arabidopsis. Here, we demonstrate that MCA1 and MCA2 are involved in a cold-induced increase in $[Ca^{2+}]_{cyt}$. The cold-induced $[Ca^{2+}]_{cyt}$ increase in *mca1* and *mca2* mutants was markedly lower than that in wild types. The *mca1 mca2* double mutant exhibited chilling and freezing sensitivity, compared to wild-type plants. Expression of *At5g61820*, *At3g51660*, and *At4g15490*, which are not regulated by the *CBF/DREB1s* transcription factor, was down-regulated in *mca1 mca2*. These results suggest that MCA1 and MCA2 are involved in the cold-induced elevation of $[Ca^{2+}]_{cyt}$, cold tolerance, and *CBF/DREB1*-independent cold signaling.

Calcium ions are used as secondary messengers in eukaryotic cells. The cytosolic Ca²⁺ concentration, $[Ca^{2+}]_{cyt}$, fluctuates in response to a variety of stimuli, including mechanical stimulation, hormones, pathogens, light, and abiotic stresses such as low temperature^{1–3}. The stimulus-specific spatiotemporal patterning of $[Ca^{2+}]_{cyt}$ dynamics is called the Ca²⁺ signature⁴, and to create these signatures, Ca²⁺ influx channels and Ca²⁺ efflux transporters that permit transient increases in $[Ca^{2+}]_{cyt}$ are required⁵.

How plant cells generate stimulus-specific Ca²⁺ signals remains unknown. To identify the spatiotemporal patterning of $[Ca^{2+}]_{cyt}$ dynamics, recombinant aequorin has been introduced as a reporter of $[Ca^{2+}]_{cyt}$ changes in plant systems⁶. In Arabidopsis plants expressing aequorin in the cytoplasm, low temperature triggers an immediate and transient rise in $[Ca^{2+}]_{cyt}$ ^{6–8}. The final temperature and cooling rate are important for sensing low temperature in Arabidopsis⁹. In mammals, many TRP (transient receptor potential) channels, which are a specific class of ion channels, function as intracellular Ca²⁺ release channels¹⁰. Some of these channels also function as thermosensors¹⁰, and TRPA1 seems to act as a sensor for cold^{11–13}. Although no proteins with high similarity to TRP channels have been identified in land-plant genomes, the genes for Cr-TRP proteins are encoded in the genomic sequence of the alga *Chlamydomonas reinhardtii* and show functional properties that are similar to those of mammalian TRP channels¹⁴.

Two Ca²⁺-permeable mechanosensitive channels, named MCA1 and MCA2 (*mid1*-complementing activity 1 and 2), have been identified in Arabidopsis^{15–19}. Both *MCA1* and *MCA2* complement deficiency of Ca²⁺ uptake in yeast cells lacking a Ca²⁺ channel composed of the Mid1 and Cch1 subunits^{15,16}. It should be noted that this complementation activity is detected under conditions that allow the Mid1/Cch1 channel to function as the sole Ca²⁺ influx system in yeast cells, suggesting that MCA1 and MCA2 can directly mediate Ca²⁺ influx in the cells lacking both Mid1 and Cch1. Electrophysiological studies have shown that both MCA1 and MCA2 produce stretch-activated currents when expressed in *Xenopus laevis* oocytes¹⁷. These results with yeast cells and *Xenopus* oocytes suggest that MCA1 and MCA2 mediate Ca²⁺ influx as mechanosensitive channels, and are not accessory

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factors that facilitate Ca^{2+} influx. Overexpression of *MCA1* enhances an increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ upon hypoosmotic shock¹⁵. The *mca2* mutant exhibits a defect in Ca^{2+} uptake from the roots¹⁶. Structurally, *MCA1* and *MCA2* have 74% identity and 89% similarity in amino acid sequences¹⁵. Both have a single transmembrane segment and an EF-hand-like motif and coiled-coil motif in the N-terminal region, as well as a plac8 motif in the C-terminal region^{15,18}. *MCA1*-GFP and *MCA2*-GFP are localized to the plasma membrane¹⁵. *MCA1* and *MCA2* form a homotrimer^{19,20}. Topological analysis has indicated that the EF-hand-like motif, the coiled-coil motif, and the plac8 motif are present in the cytoplasm¹⁸, suggesting that both channels recognize intracellular Ca^{2+} . The *MCA* genes are conserved in the plant kingdom²¹, and an increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ as a result of hypo-osmotic shock is mediated by *MCA* proteins in rice and tobacco^{22,23}.

Application of the patch-clamp technique has demonstrated that Ca^{2+} -permeable channels are transiently activated by cold shock in *Arabidopsis* mesophyll cells⁷. In plants, extracellular freezing causes dehydration and mechanical stresses on the plasma membrane, and cold-acclimated plant plasma membranes become resistant to mechanical stress²⁴. Expression of *CBF2* is induced not only by cold, but also by mechanical stress²⁵. Therefore, it is assumed that mechanical stress may be one of the factors involved in cold acclimation.

Three *CBF/DREB1* (C-repeat binding factor/DRE binding factor 1) transcription factors have been extensively studied. They belong to the AP2/ERF (Apetala/ethylene-responsive factor) superfamily and are important factors for cold acclimation in plants²⁶. *CBF/DREB1* genes are rapidly and transiently induced after cold treatment²⁷, and overexpression of *CBF/DREB1* constitutively enhances freezing tolerance^{28,29}. Under cold stress, *CBF/DREB1* proteins bind to *CRT/DRE cis*-elements in the promoter of cold-regulated (*COR*) genes and induce transcription²⁸. However, gene expression analyses reveals that only 6.5% of the total *COR* genes are regulated by *CBF/DREB1*³⁰. In addition to *CBF/DREB1* genes, 27 transcription factors that were up-regulated at an early stage after cold treatment were considered as first-wave transcription factors³⁰. Use of the *cbf1/2/3* triple mutant showed that six first-wave transcription factors are partially regulated by *CBF/DREB1*, whereas the transcription factors *HSFC1*, *ZAT12*, and *CZF1*, which regulate cold-regulated genes^{30,31}, are not regulated by *CBF/DREB1*³². As acclimated *cbf1/2/3* triple mutants are more tolerant of freezing stress than non-acclimated ones³³, and the expression of a large number of cold-regulated genes is not affected by the *cbf1/2/3* triple mutation³², a *CBF/DREB1*-independent pathway may control cold tolerance. Overexpression of *HSFC1* enhances cold tolerance without an increase in expression of *CBF1*, *CBF2*, or *CBF3*³⁰, suggesting that *HSFC1* is one of the important transcription factors controlling non-*CBF/DREB1* regulons and cold tolerance.

Here, we demonstrate that *MCA1* and *MCA2* are involved in a transient rise in $[\text{Ca}^{2+}]_{\text{cyt}}$ upon cold shock. The cold-induced increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ was smaller in the *mca1* and *mca2* mutants than in the Col-0 wild type. The *mca1 mca2* double mutant exhibited increased sensitivity to chilling and freezing stresses. These results suggest that *MCA1* and *MCA2* are involved in cold-induced Ca^{2+} influx and that the reduced $[\text{Ca}^{2+}]_{\text{cyt}}$ increase caused by the *mca1* and *mca2* mutations affects cold acclimation. As the *CBF/DREB1* genes and their regulon genes were not down-regulated in the *mca1 mca2* mutant, *MCA* may not be involved in the regulation of *CBF/DREB1*-dependent cold signaling.

Results

***MCA1* and *MCA2* are involved in a cold-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ increase.** To monitor changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ (the cytosolic concentration of Ca^{2+}), *Arabidopsis* seedlings expressing aequorin, a Ca^{2+} indicator¹⁵, that had been immersed in MS medium (400 μl) at 22 °C were exposed to low temperatures by the addition of MS medium (500 μl) kept at 3, 10, or 22 °C. $[\text{Ca}^{2+}]_{\text{cyt}}$ in the wild type was significantly increased by a 3 °C shock (Fig. 1A green line and B), moderately by a 10 °C shock (Fig. 1A black line and C), and just a little by a 22 °C shock (Fig. 1A red line and D). On the other hand, the magnitude of the cold-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ increase was markedly lower in the *mca1*, *mca2*, and *mca1 mca2* mutants (Fig. 1). Small increases observed in response to the 22 °C shock in both the wild type and the mutants could be a consequence of mechanical stress rather than cold stress, because MS medium (a fluid) was added to induce the response. These results suggest that *MCA1* and *MCA2* contribute to a $[\text{Ca}^{2+}]_{\text{cyt}}$ increase upon cold shock.

Since *MCA1* and *MCA2* are present in the plasma membrane^{15,16}, the cold-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ increase could be brought about by Ca^{2+} influx. To examine this possibility, we preincubated seedlings for 30 min in MS medium including either a Ca^{2+} chelator, EGTA, or a plasma membrane ion channel blocker, La^{3+} or Gd^{3+} , and then monitored changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ upon cold shock. As expected, the cold-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ increase was inhibited by EGTA (Fig. 2A,B), La^{3+} (Fig. 2C,D), and Gd^{3+} (Fig. 2E,F) in the wild type and in all the *mca* mutants, although the inhibition rates of the wild type were greater than those of the *mca* mutants. It should also be noted that significant $[\text{Ca}^{2+}]_{\text{cyt}}$ increases remained in all the *mca* mutants, as well as in the wild type, suggesting that there is another cold-induced Ca^{2+} transport system(s) that is insensitive to the blockers we used in the plasma membrane, or that is in the intracellular compartment.

Even though the *mca* mutants exhibited a reduced cold-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ increase, the mutants looked healthy when they grew under normal conditions (Fig. 3A). To examine whether the *mca* mutation affects plant growth under normal conditions, fresh weight and chlorophyll contents were measured (Fig. 3B,C). The *mca1*, *mca2*, and *mca1 mca2* plants had similar values, as did the wild type, suggesting that plant development in the aerial part is unaffected by *MCA1* and *MCA2*.

Mutations in *MCA1* and *MCA2* result in cold sensitivity. Since the *mca* mutants exhibited a reduced cold-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ increase, we investigated their cold sensitivity. Three-week-old plants were incubated at 4 °C for 1 week to acclimate to cold stress. These plants were then exposed to freezing temperatures (Fig. 4A,B). Before this exposure, *mca1*, *mca2*, *mca1 mca2* and the wild-type plants looked healthy (Fig. 3A). After this exposure, the survival of the *mca1* mutant was similar to that of the wild type. On the other hand, the *mca2* mutant exhibited a freezing-sensitive phenotype (Fig. 4A,B). Furthermore, the *mca1 mca2* double mutant was more

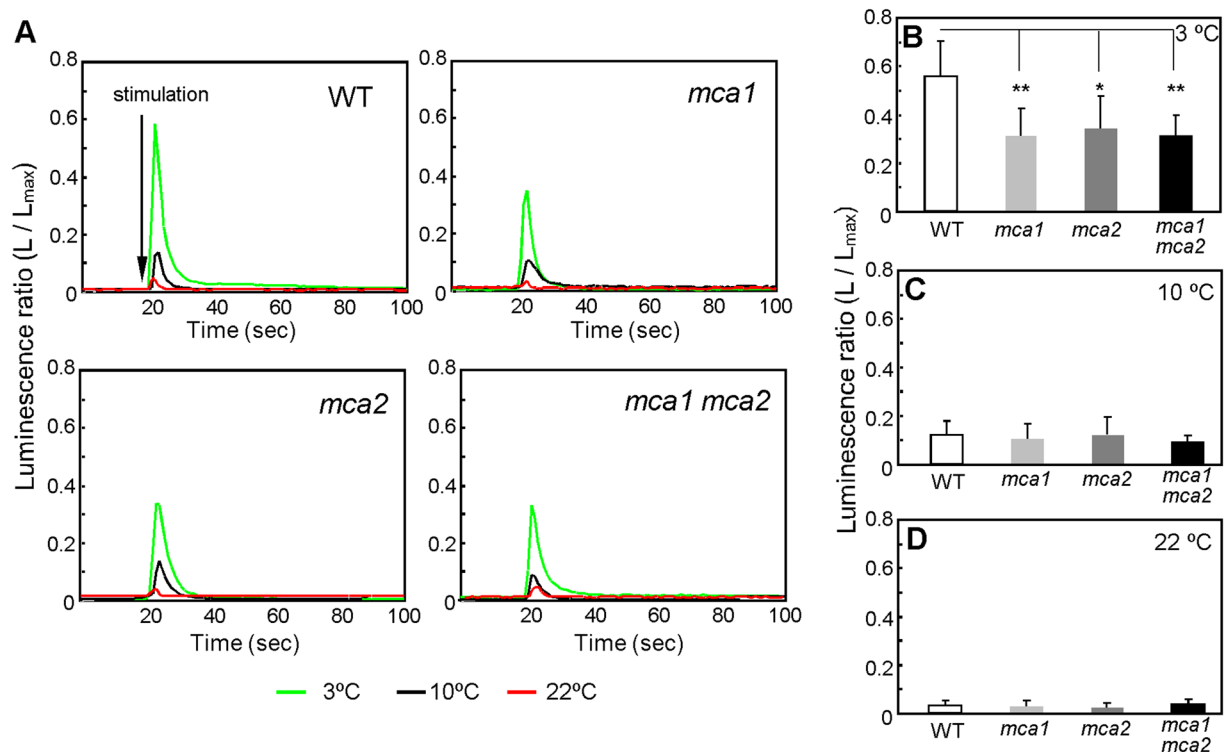


Figure 1. Transient cold-induced increase in cytosolic Ca²⁺ is lower as a result of the *mca* mutation. (A) Relative luminescence of plants harboring aequorin was measured before and after the addition (indicated by the vertical arrow) of precooled solution (3 °C or 10 °C) or room temperature solution (22 °C). The figures are of representative data. The peak luminescence after the addition of solution at 3 °C (B, $n \geq 10$), 10 °C (C, $n \geq 9$), and 22 °C (D, $n \geq 17$) is shown. Data represent the means \pm SD. n indicates the number of seedlings. * $p < 0.05$; ** $p < 0.005$ versus the wild type. Significance was determined using unpaired Student's t tests.

sensitive to freezing stresses than the *mca2* mutant (Fig. 4A,B). Electrolyte leakage from the *mca1 mca2* double mutant was much higher (approximately 50%) than that of the *mca* single mutants and the wild type, even before it was subjected to freezing temperatures, and it increased as the freezing temperature was lowered (Fig. 4C). At every freezing temperature we employed (−3 to −9 °C), the leakage was greatest in the double mutant. To confirm whether the freezing sensitivity of the *mca1 mca2* mutant was caused by the mutation in *MCA1* or *MCA2*, complement lines were produced. *MCA1pro::MCA1* or *MCA2pro::MCA2* was expressed in the *mca1 mca2* mutant (Fig. 4D,E). Because the own promoter was used for expression of *MCA1* or *MCA2*, the expression level of *MCA1* in the complement lines, *MCA1pro::MCA1* in *mca1 mca2*, was similar to that of wild type and the *mca2* mutant (Figure S1). The expression level of *MCA2* in the complement lines, *MCA2pro::MCA2* in *mca1 mca2*, was slightly higher than that of WT and *mca1* (Figure S1). The sensitivity of *MCA2pro::MCA2*-expressing *mca1 mca2* mutant was recovered (Fig. 4E). On the other hand, the survival ratio of *MCA1pro::MCA1*-expressing *mca1 mca2* was similar to that of *mca2* (Fig. 4E). The wild-type, *mca1*, *mca2*, and *mca1 mca2* plants without cold acclimation were also treated with a freezing temperature for 1 h (Fig. 4F). Before acclimation, the cold sensitivity of the *mca1 mca2* plants was slightly greater than that of wild-type, *mca1* and *mca2* plants (Fig. 4G). Furthermore, electrolyte leakage of the *mca1 mca2* mutant was a little higher than that of the wild type (Fig. 4H). These results suggest that *MCA* mainly functions in the regulation of cold tolerance during cold acclimation.

To examine whether the mutant exhibits chilling sensitivity, wild-type, *mca1*, *mca2*, and *mca1 mca2* plants were incubated at 4 °C for 1 month under continuous light conditions. The leaves of the *mca1 mca2* double mutant looked unhealthy (Fig. 4I). Thus, to quantify chilling sensitivity, chlorophyll content was measured. The chlorophyll content in the *mca1 mca2* double mutant was approximately three-fourths that of the wild type (Fig. 4J). No detectable difference was observed between the wild type and the *mca* single mutants. These results suggest that the double mutation in *MCA1* and *MCA2* results in hypersensitivity to cold stress in *Arabidopsis* plants.

Down-regulation of cold-inducible genes is governed by a non-*CBF/DREB1* regulon. The expression of the *CBF/DREB1* genes and their regulon genes, *COR15A*, *COR47*, and *RD29A*, was investigated. To perform this, three-week-old wild-type, *mca1*, *mca2*, and *mca1 mca2* plants were exposed to cold at 4 °C for appropriate periods, and RNA prepared from the plants was subjected to a quantitative RT-PCR analysis. Interestingly, the expression of these genes was slightly up-regulated in the *mca1 mca2* double mutant, especially soon after the start of the cold treatment (Fig. 5A). This increase could be the consequence of a compensatory response caused by a lack of the function of *MCA1* and *MCA2* and suggests that both proteins may control another cold signaling pathway. *CBF/DREB1* and its regulatory genes are only partly responsible for the acquisition of tolerance to

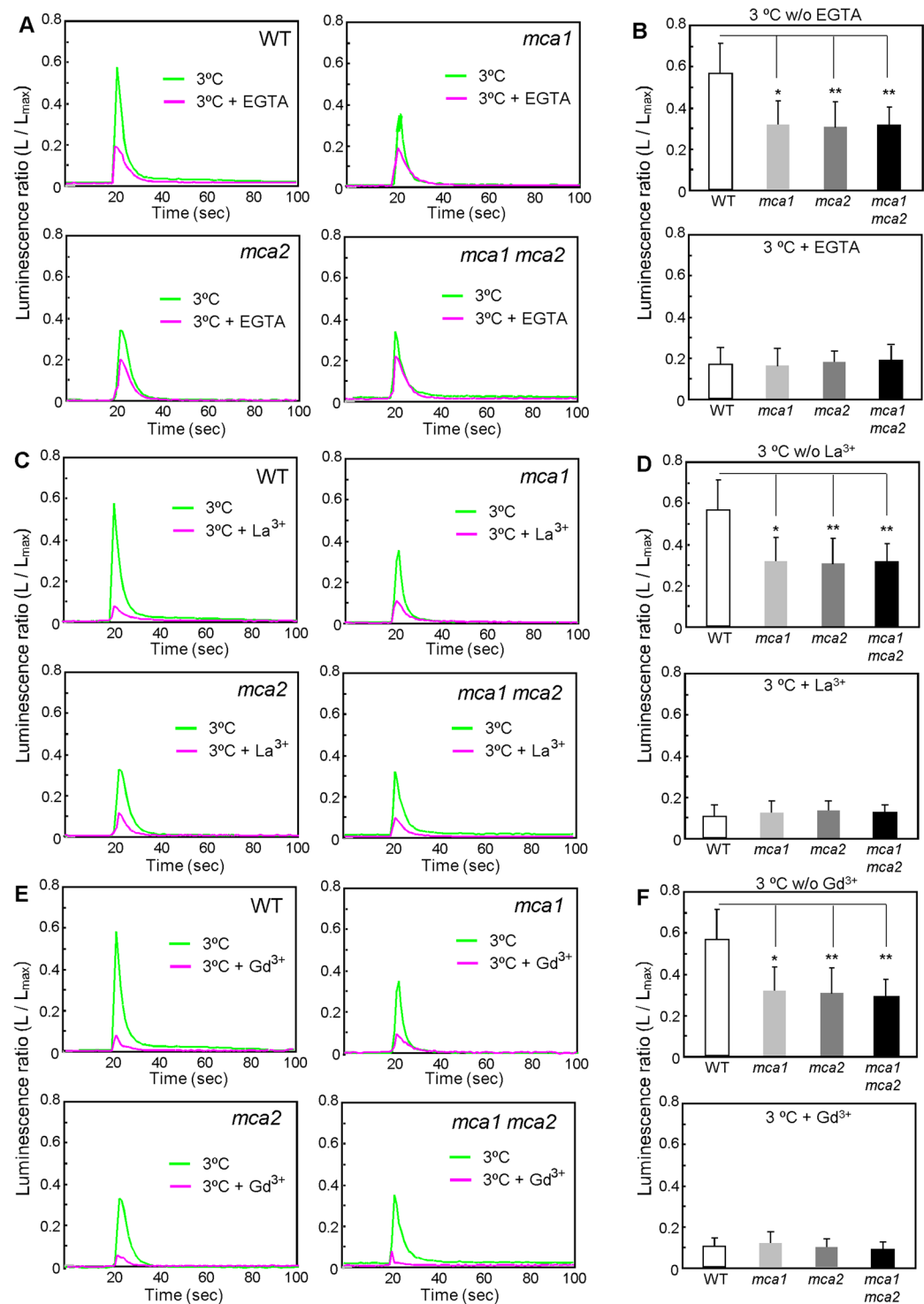


Figure 2. Effect of channel blockers and a Ca^{2+} chelator on the cold-shock-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ increase. Thirty minutes before cold shock, 5 mM EGTA (A), 1 mM La^{3+} (C), or 1 mM Gd^{3+} (D) was added to the medium. Then, the relative luminescence of a plant harboring aequorin was measured, as in Fig. 1, before and after the application of precooled solution (3°C). The peak luminescence after the addition of the solution at 3°C with or without 5 mM EGTA (B, $n \geq 17$), 1 mM La^{3+} (D, $n \geq 9$), or 1 mM Gd^{3+} (F, $n \geq 9$) is shown. Data represent the means \pm SD. * $p < 0.05$; ** $p < 0.005$ versus the wild type in each treatment. Significance was determined using unpaired Student's t tests.

freezing stress for cold acclimation³⁰. Therefore, we examined the expression of cold-inducible genes that are governed by *H5FC1* but not by *CBF2*³⁰, such as *At5g61820*, *At3g51660*, and *At4g15490*, which encode an unknown protein, a tautomerase/MIF superfamily protein, and a UDP-glycosyltransferase superfamily protein, respectively.

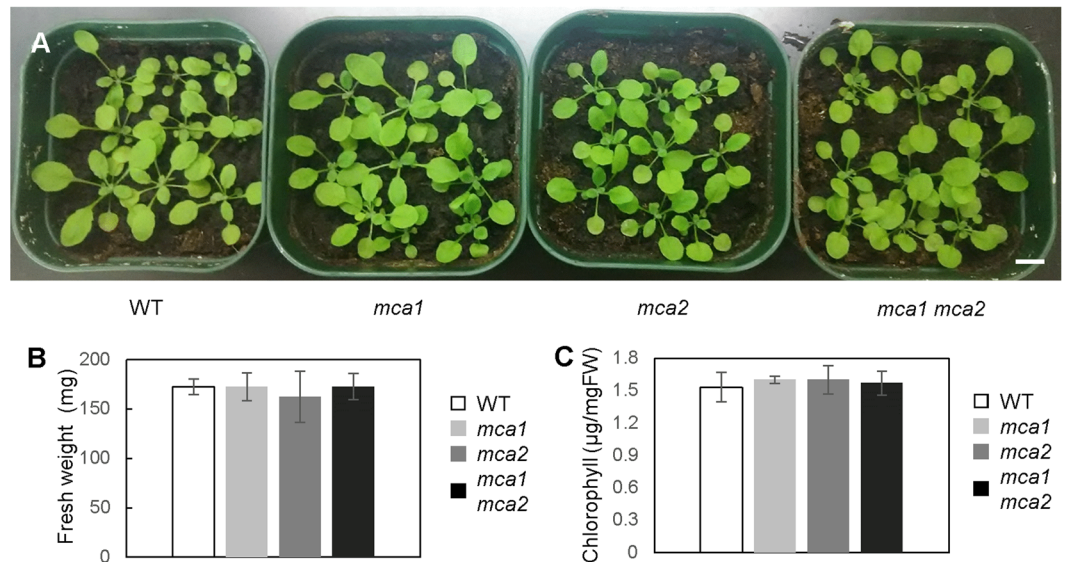


Figure 3. The growth of the *mca1 mca2* mutant is normal at room temperature. (A) Representative 3-week old plants of wild type, *mca1*, *mca2*, and *mca1 mca2* mutants (nine plants in each pot) are displayed. The bar indicates 1-cm length. Fresh weight (B) and chlorophyll content (C) of each plant were measured. Values represent the means \pm SD ($n = 10$). No significant difference was observed with an unpaired Student's *t*-test.

Figure 5B shows that the expression of the three genes was significantly down-regulated in the *mca1 mca2* double mutant. Expression of MCA1 and MCA2 themselves was unchanged upon cold shock (Fig. 5C). According to these results, it is plausible that MCA1 and MCA2 mediate cold tolerance by participating in a pathway other than the *CBF/DREB1* pathway.

Discussion

In the present study, we have demonstrated that MCA1 and MCA2 play a role in the transient rise in $[Ca^{2+}]_{cyt}$ upon cold shock and are also involved in chilling and freezing tolerance. The *mca1 mca2* double mutant exhibited a lower cold-induced increase in $[Ca^{2+}]_{cyt}$ than the wild type (Fig. 1), as well as an increased sensitivity to cold stress (Fig. 4). Although the *mca1* and *mca2* single mutants exhibited a lower cold-induced increase in $[Ca^{2+}]_{cyt}$ like the double mutant, the *mca1* single mutant did not show a cold sensitive phenotype similar to that of the *mca2* single and *mca1 mca2* double mutants. We speculate that a reason for this discrepancy may be a difference in the spatial expression patterns of MCA1 and MCA2 in Arabidopsis plants¹⁶, as explained in more detail in a later paragraph. As for the regulation of gene expression, *CBF/DREB1* genes and their regulon genes were not down-regulated in the *mca1 mca2* double mutant (Fig. 5), suggesting that MCA may not regulate *CBF/DREB1*-dependent cold signaling.

Different stimuli produce different patterns of Ca^{2+} elevation and oscillations with different frequencies, and these are called Ca^{2+} signatures. As shown in Fig. 1, low-temperature stress stimulates a transient increase in $[Ca^{2+}]_{cyt}$ ³⁴. MCA1 and MCA2 have been identified as plasma membrane proteins involved in Ca^{2+} influx in response to mechanical stimuli, such as touch, gravity, flexure, and turgor^{15,16}. In the *mca1* or *mca2* mutant, the magnitude of the cold-induced $[Ca^{2+}]_{cyt}$ increase was lower, by approximately 40%, than that in the wild type (Fig. 1). These results suggest that MCA1 and MCA2 are partially involved in Ca^{2+} influx in response to cold shock. Application of a mechanosensitive Ca^{2+} channel blocker, Gd^{3+} , prevents the induction of cold-regulated genes³⁵. As two Ca^{2+} channel inhibitors, La^{3+} and Gd^{3+} , still reduced the cold-induced $[Ca^{2+}]_{cyt}$ increase in the *mca* mutants, and the *mca* mutations were unable to block $[Ca^{2+}]_{cyt}$ increases completely (Fig. 2), other cold-activatable Ca^{2+} transport system(s) must exist in the plasma membrane and/or organellar membranes. Indeed, it is reported that the vacuole, the major intracellular Ca^{2+} store, is involved in a cold-induced Ca^{2+} release⁸. In the present study, we did not calibrate the bioluminescent intensity of aequorin for $[Ca^{2+}]_{cyt}$ because of difficulties in the precise calibration, although we noted a report describing a successful calibration specific for the isoform of aequorin and temperature that the authors used⁸. It should be mentioned that although the present study has clearly suggested the involvement of MCA1 and MCA2 in cold-induced $[Ca^{2+}]_{cyt}$ increases, it remains to be examined whether complementation lines of *mca1*, *mca2* and *mca1 mca2* mutants expressing aequorin show a wild-type level of cold-induced $[Ca^{2+}]_{cyt}$ increases.

Plants employ several kinds of mechanisms to control Ca^{2+} -regulated gene expression³⁶. However, it is still unclear how cold-induced $[Ca^{2+}]_{cyt}$ increases are recognized. One possible mechanism involves calmodulin-binding transcription factors (CAMTAs). CAMTAs possess calmodulin (CaM)-binding domains³⁷ and CAMTAs play a role in the regulation of gene expression in response to Ca^{2+} signals³⁸. CAMTA3 is a positive regulator of *CBF2/DREB1C* expression and binds to the consensus sequence of a CGCG core motif, a *cis*-element for CAMTAs, in the promoter of *CBF2/DREB1C*³⁹. The *camta2 camta3* double mutant is sensitive to

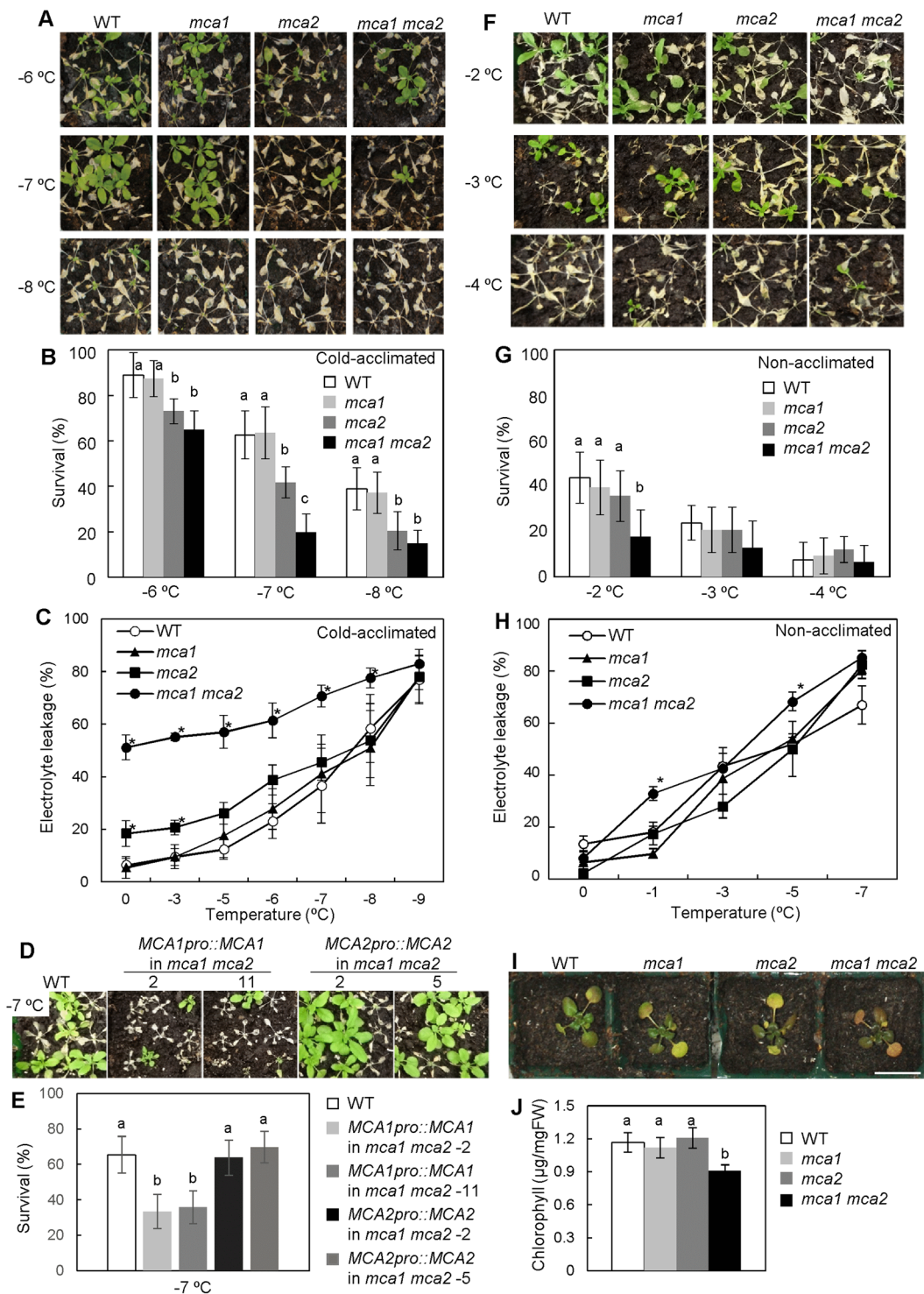


Figure 4. The *mca1 mca2* double mutant exhibited sensitivity to cold stress. **(A)** Freezing sensitivity of the *mca1 mca2* double mutant after cold acclimation. Three-week-old plants were incubated at 4 °C for 1 week, then plants were used for freezing treatment. Photographs are representative plants 7 days after 4-h exposure to the indicated temperature. **(B)** Survival rates were determined for 9 plants after freezing treatment at the indicated temperature. The survival ratio was calculated from 9 plants per pots. Data represent the means \pm SD calculated from the data of 9 independent experiments. Differences between the values of each treatment were evaluated by one-way ANOVA followed by the Tukey-Kramer test. Difference of alphabet letters at each temperature indicates statistically significant difference ($p < 0.05$). **(C)** Electrolyte leakage from cold-acclimated wild-type, *mca1*, *mca2*, and *mca1 mca2* plants after exposure to the indicated temperature (programmed to cool at a rate of 2 °C h⁻¹). Data represent the means \pm SD ($n = 4$ leaves, each from a different plant). * $p < 0.05$ compared with the value of wild type at each temperature (one-way ANOVA followed by the Tukey-Kramer test). Data were representative experiments from 3 biological independent experiments. **(D)** Freezing sensitivity of the *mca1*

mca2 double mutant harboring *MCA1pro::MCA1* (lines #2 and #11) or *MCA2pro::MCA2* (lines #2 and #5). Photographs are representative plants 7 days after 4-h exposure to -7°C . (E) The survival ratio at -7°C was calculated from 9 plants per pots. Data represent the means \pm SD calculated from the data of 9 independent experiments. Difference between values of each treatment were evaluated by one-way ANOVA followed by the Tukey-Kramer test. Difference of alphabet letters at each temperature indicates statistically significant difference ($p < 0.05$). (F) Freezing sensitivity of the *mca1 mca2* double mutant without cold acclimation. Three-week-old plants were treated with a freezing temperature. Photographs are representative plants after freezing treatment. Photographs are representative plants 7 days after 1-h exposure to the indicated temperature. (G) The survival ratio was calculated from 9 plants per pots. Difference between values of each treatment were evaluated by one-way ANOVA followed by the Tukey-Kramer test. Difference of alphabet letters at each temperature indicates statistically significant difference ($p < 0.05$). (H) Electrolyte leakage from non-acclimated wild-type, *mca1*, *mca2*, and *mca1 mca2* plants after exposure to the indicated temperature. Data represent the means \pm SD ($n = 3$ leaves, each from a different plant). $*p < 0.05$ compared with the value of wild types at each temperature (ANOVA followed by the Tukey-Kramer test). (I) Photographs are of representative wild type, *mca1*, *mca2*, and *mca1 mca2* mutants after incubation at 4°C for 1 month. Five-day-old plants were incubated at 4°C for 1 month. (J) The chlorophyll content of the plants was determined. Values represent the means \pm SD ($n = 7$ plants per each genotype). Difference of alphabet letters indicates a significant difference ($p < 0.05$) as determined by one-way ANOVA followed by the Tukey-Kramer test.

freezing temperatures³⁹. Microarray analyses demonstrated that the expression level of *HSFC1* (*At3g24520*) in the *camta1/2/3* mutant is lower than that in the wild type⁴⁰. CAMTA is one of possibilities how increased $[\text{Ca}^{2+}]_{\text{cyt}}$ is recognized.

As shown in Fig. 1, the *mca1 mca2* double mutation reduced about 40% of the $[\text{Ca}^{2+}]_{\text{cyt}}$ increase. The transient increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ may be conducted by other Ca^{2+} -permeable channels that are responsible for 60% of the transient increase. One such channel could be AtGLR3.4, a member of the Arabidopsis homologs of ionotropic glutamate receptors, whose expression is up-regulated under cold stress⁴¹. Another such channel could be the cyclic nucleotide-gated ion channel (CNGC) family. In rice, the expressions of 10 out of 16 CNGC genes are induced under cold stress⁴².

The *mca1 mca2* double mutant exhibited increased sensitivity to chilling and freezing stresses, even though the single mutants did not exhibit a severe phenotype (Fig. 4). Both MCA1 and MCA2 complement a Ca^{2+} uptake deficiency of yeast cells lacking a Ca^{2+} channel composed of Mid1 and Cch1^{15,16}, and generate stretch-activated currents in *Xenopus* oocytes¹⁷. Even though MCA1 and MCA2 have similar functions as Ca^{2+} -permeable mechanosensitive channels, their spatial expression patterns are not necessarily the same in whole plants¹⁶. *MCA1p::GUS* and *MCA2p::GUS* are expressed in vascular tissues of cotyledons, leaves and primary roots in common. On the other hand, *MCA1p::GUS* is expressed in the promeristem and adjacent elongation zone of the primary root, while *MCA2p::GUS* is not. *MCA2p::GUS* is expressed in mesophyll cells of cotyledons and leaves, but *MCA1p::GUS* is not. In addition, *MCA2p::GUS* is expressed more than *MCA1p::GUS* at the center of rosettes in a region corresponding to the shoot apical meristem. To survive freezing stress, the shoot apical meristem should be protected to recover plant growth⁴³. Based on the observation of differences in the spatial expression patterns of *MCA1p::GUS* and *MCA2p::GUS* in whole plants, it is possible to speculate that the differences may allocate MCA1 and MCA2 a role in the acquisition of tolerance to cold stress. This allocation could explain why only the double mutant becomes hypersensitive to chilling and freezing stresses.

According to microarray analyses, several genes were up-regulated in *HSFC1*-overexpressed plants, but not in *CBF2*-overexpressed plants³⁰. Some *HSFC1*-dependent and *CBF2*-independent cold-regulated genes, such as *At5g61820*, *At3g51660*, and *At4g15490*, encoding an unknown protein, a tautomerase/MIF superfamily protein, and the UDP-glycosyltransferase superfamily protein UGT84A3, respectively, were down-regulated in the *mca1 mca2* double mutant (Fig. 5). It is possible that the MCA1/2-regulated Ca^{2+} signal is transduced to a *HSFC1*-dependent pathway to enhance cold tolerance. This possibility warrants further study.

In conclusion, two mechanosensitive Ca^{2+} channels, MCA1 and MCA2, are involved in a cold-induced transient $[\text{Ca}^{2+}]_{\text{cyt}}$ increase in Arabidopsis, and in the regulation of cold tolerance through a pathway other than the *CBF/DREB1*-dependent pathway.

Methods

Plant materials. The Columbia-0 (Col-0) of Arabidopsis and its isogenic, transgenic lines *mca1*-null, *mca2*-null, and *mca1*-null *mca2*-null were previously described^{15,16}. The complementation lines *MCA1pro::MCA1* in *mca1*-null *mca2*-null and *MCA2pro::MCA2* in *mca1*-null *mca2*-null were also previously described^{15,16}.

Monitoring of $[\text{Ca}^{2+}]_{\text{cyt}}$ changes following cold shock treatment. Apoaquorin-expressing seedlings grown at 22°C on MS medium supplemented with 0.8% agar and 1% sucrose under 16-h light conditions at $40\text{--}60\ \mu\text{M m}^{-2}\ \text{s}^{-1}$ light intensity were used to monitor $[\text{Ca}^{2+}]_{\text{cyt}}$ changes upon cold shock. A seedling was harvested 14 days after sowing and incubated overnight at 22°C in 2 ml of MS medium containing $2.5\ \mu\text{M}$ coelenterazine in the dark to reconstitute aequorin. The seedling was transferred to fresh MS medium (400 μl) kept at 22°C in a tube (Microtech-Nition, #NU-063, Funabashi, Japan) and received an additional 500 μl of the same medium kept at 3, 10, or 22°C . Luminescence (L) from aequorin in the whole seedlings was measured using a luminometer (Microtech-Nition, Model NU-2500). At the end of each monitoring, 1 ml of 20% ethanol/2 M CaCl_2 solution was added to the medium (0.9 ml total) to measure the maximum luminescence (L_{max}). The luminescence ratios (L/L_{max}) are presented in Figs 1 and 2.

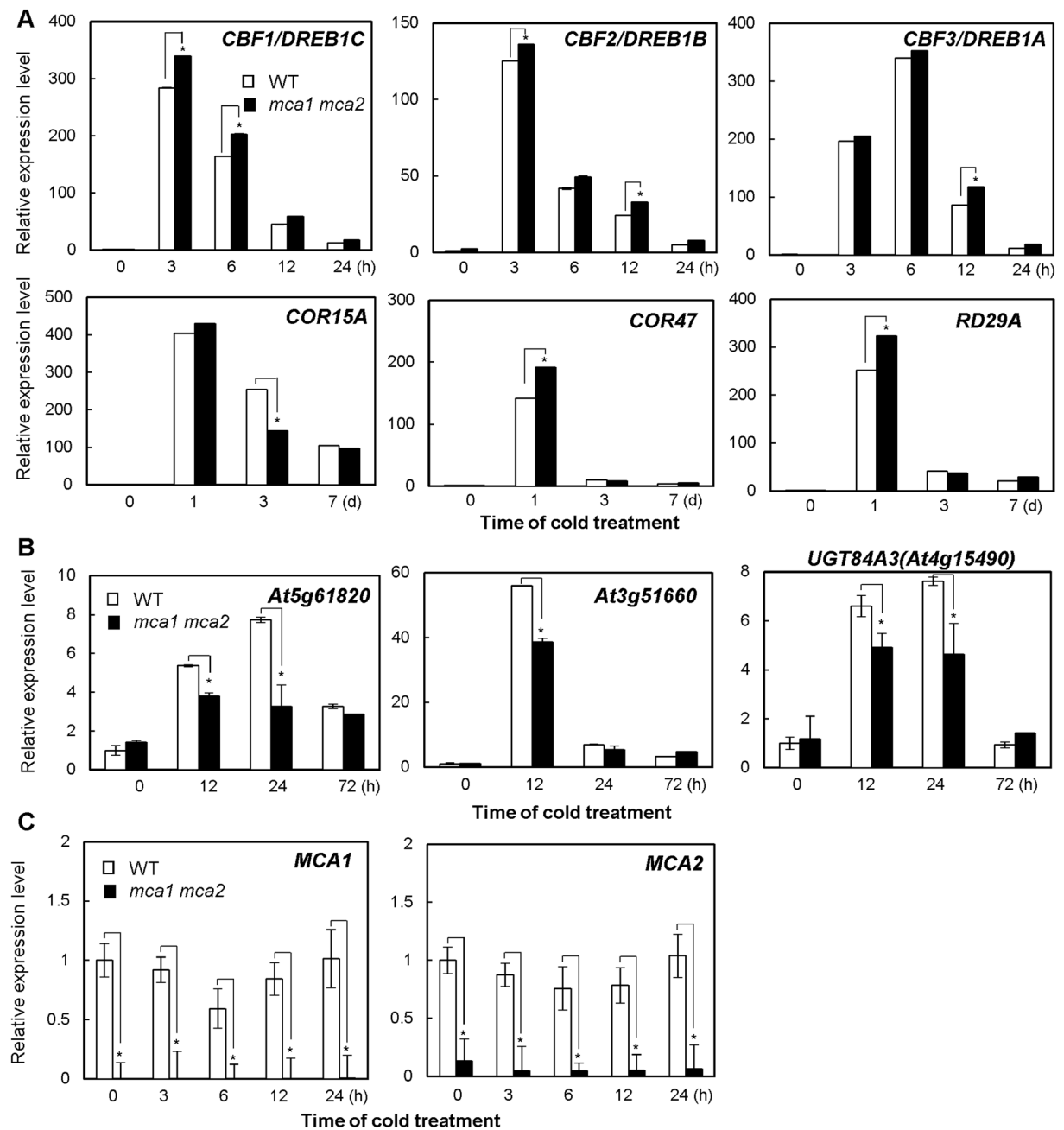


Figure 5. The expression levels of several cold-regulated genes, which are not regulated by *CBF/DREB1*, were reduced in the *mca1 mca2* double mutant. (A) Relative mRNA transcript levels of *CBF/DREB1* and its regulon genes, *COR15A*, *COR47*, and *RD29A*, in wild-type and *mca1 mca2* plants were determined by quantitative RT-PCR analyses. Three-week-old plants grown at 24°C were incubated at 4°C for the indicated time. Values represent the means \pm SD ($n = 3$) from representative experiments from 3 biologically independent experiments. (B) Relative expression levels of cold-regulated genes that are not *CBF/DREB1*-regulon genes³⁰ were determined by quantitative RT-PCR analyses. Values represent the means \pm SD ($n = 3$) from representative experiments from 3 biologically independent experiments. (C) The expression levels of *MCA1* and *MCA2* in wild types and *mca1 mca2* double mutants were investigated by quantitative RT-PCR analyses. An asterisk indicates a significant difference from wild-type plants at each point ($p < 0.05$) as determined by unpaired Student's *t*-tests.

Plant freezing and chilling assay. Wild-type (ecotype Col-0), *mca1*, *mca2*, and *mca1 mca2* plants were grown at 24°C for 3 weeks in soil with fluorescent lighting (16 h/8 h light/dark photoperiod). These plants were then incubated at 4°C for 1 week for acclimation to low temperatures. For non-acclimation, 3-week-old plants were treated with the freezing temperature without incubation at 4°C. Whole-plant freezing assays were performed as previously described⁴⁴. Briefly, plants were incubated at 0°C for 1 h, and the temperature was lowered by 2°C h⁻¹ until it reached to the indicated temperature, and then held at the desired temperature for 1 h or 4 h

for non-acclimated plants or cold-acclimated plants, respectively, in the incubator (IN602, Yamato Scientific Co., Ltd., Tokyo, Japan). After cold acclimation, the plants were incubated at 4 °C overnight and transferred to 24 °C. The survival ratio was determined 1 week after the freezing test.

For the chilling assay, 5-day-old plants were incubated at 4 °C. After incubation for 1 month under constant illumination, the chlorophyll content of the plants was determined. Eighty percent acetone was added to leaves ground with liquid nitrogen. The mixture was shaken at 4 °C. After centrifugation, the absorbances of the supernatant at 663 nm and 646 nm were measured. Total chlorophyll content was calculated as $17.3 A_{663} + 7.18 A_{646}$ ⁴⁵.

Electrolyte leakage from fully developed rosettes of leaves of three-week-old plants was measured as previously described^{46,47}. The sample was incubated in a refrigerated circular bath (TRL-11P, Thomas Kagaku Kikai, Co., Ltd., Japan). The conductivity was measured with a conductivity meter (CD-4302, Lutron Electronic Enterprise Co., Ltd., Taipei, Taiwan).

RNA preparation and quantitative RT-PCR. Three-week-old wild-type (ecotype Col-0), *mca1*, *mca2*, and *mca1 mca2* plants were subjected to cold treatment at 4 °C for the indicated time. Isolation of total RNA, cDNA synthesis, and quantitative RT-PCR were performed as previously described⁴⁷. The primers used to detect *CBF/DREB1* and its regulon genes were also previously described⁴⁷. Other genes were detected with gene-specific primers for *At5g61820* (5'-GAGGCACCTGCGAGAAGCTTGAG-3' and 5' GTAACCATCTTCCCGTTTCTGTGTC-3'), *At3g51660* (5'-GACCTCAAAACTTAGTGATGGTG-3' and 5'-TTAACTTGTTTGGTGATGCCTCC-3'), *At4g15490* (5'-CCTCCATGGAAGGGACATTTGTAGA-3' and 5'-ACAAGCAATCGCAGGATGAGCCA-3'), *MCA1* (5'-AAGATTGCCACTGCAGCATCC-3' and 5'-ACGCCATTAGCTCATTACATGCTTC-3'), and *MCA2* (5'-AAGATCATTGCAACACCGTGGA-3' and 5'-GTGTCTCAAGCAAAGACAAGTTTC-3').

References

- Sanders, D., Pelloux, J., Brownlee, C. & Harper, J. F. Calcium at the crossroads of signaling. *Plant Cell* **14**, s401–417 (2002).
- Dodd, A. N., Kudla, J. & Sanders, D. The language of calcium signaling. *Annu. Rev. Plant Biol.* **61**, 593–620 (2010).
- White, P. J. & Broadley, M. R. Calcium in plants. *Ann. Bot.* **92**, 487–511 (2003).
- Monshausen, G. B. Visualizing Ca²⁺ signatures in plants. *Curr. Opin. Plant Biol.* **15**, 677–682 (2012).
- McAinsh, M. R. & Pittman, J. K. Shaping the calcium signature. *New Phytol.* **181**, 275–294 (2009).
- Knight, M. R., Campbell, A. K., Smith, S. M. & Trewavas, A. J. Transgenic plant aequorin reports the effects of touch and cold-shock and elicitors on cytoplasmic calcium. *Nature* **352**, 524–526 (1991).
- Carpaneto, A. *et al.* Cold transiently activates calcium-permeable channels in Arabidopsis mesophyll cells. *Plant Physiol.* **143**, 487–494 (2007).
- Knight, H., Trewavas, A. J. & Knight, M. R. Cold calcium signaling in Arabidopsis involves two cellular pools and a change in calcium signature after acclimation. *Plant Cell* **8**, 489–503 (1996).
- Knight, M. R. Signal transduction leading to low-temperature tolerance in *Arabidopsis thaliana*. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **357**, 871–875 (2002).
- Gees, M., Colsoul, B. & Nilius, B. The role of transient receptor potential cation channels in Ca²⁺ signaling. *Cold Spring Harb. Perspect. Biol.* **2**, a003962 (2010).
- Karashima, Y. *et al.* TRPA1 acts as a cold sensor *in vitro* and *in vivo*. *Proc. Natl. Acad. Sci. USA* **106**, 1273–1278 (2009).
- Aubdool, A. A. *et al.* TRPA1 is essential for the vascular response to environmental cold exposure. *Nat. Commun.* **5**, 5732 (2014).
- del Camino, D. *et al.* TRPA1 contributes to cold hypersensitivity. *J. Neurosci.* **30**, 15165–15174 (2010).
- Arias-Darraz, L. *et al.* A transient receptor potential ion channel in *Chlamydomonas* shares key features with sensory transduction-associated TRP channels in mammals. *Plant Cell* **27**, 177–188 (2015).
- Nakagawa, Y. *et al.* Arabidopsis plasma membrane protein crucial for Ca²⁺ influx and touch sensing in roots. *Proc. Natl. Acad. Sci. USA* **104**, 3639–3644 (2007).
- Yamanaka, T. *et al.* MCA1 and MCA2 that mediate Ca²⁺ uptake have distinct and overlapping roles in Arabidopsis. *Plant Physiol.* **152**, 1284–1296 (2010).
- Furuichi, T., Iida, H., Sokabe, M. & Tatsumi, H. Expression of Arabidopsis MCA1 enhanced mechanosensitive channel activity in the *Xenopus laevis* oocyte plasma membrane. *Plant Signal. Behav.* **7**, 1022–1026 (2012).
- Kamano, S. *et al.* Transmembrane topologies of Ca²⁺-permeable mechanosensitive channels MCA1 and MCA2 in *Arabidopsis thaliana*. *J. Biol. Chem.* **290**, 30901–30909 (2015).
- Nakano, M., Iida, K., Nyunoya, H. & Iida, H. Determination of structural regions important for Ca²⁺ uptake activity in Arabidopsis MCA1 and MCA2 expressed in yeast. *Plant Cell Physiol.* **52**, 1915–1930 (2011).
- Shigematsu, H. *et al.* Structural characterization of the mechanosensitive channel candidate MCA2 from *Arabidopsis thaliana*. *PLoS ONE* **9**, e87724 (2014).
- Kurusu, T., Kuchitsu, K., Nakano, M., Nakayama, Y. & Iida, H. Plant mechanosensing and Ca²⁺ transport. *Trends Plant Sci.* **18**, 227–233 (2013).
- Kurusu, T. *et al.* Plasma membrane protein OsMCA1 is involved in regulation of hypo-osmotic shock-induced Ca²⁺ influx and modulates generation of reactive oxygen species in cultured rice cells. *BMC Plant Biol.* **12**, 11 (2012).
- Kurusu, T. *et al.* Involvement of the putative Ca²⁺-permeable mechanosensitive channels, NtMCA1 and NtMCA2, in Ca²⁺ uptake, Ca²⁺-dependent cell proliferation and mechanical stress-induced gene expression in tobacco (*Nicotiana tabacum*) BY-2 cells. *J. Plant Res.* **125**, 555–568 (2012).
- Yamazaki, T., Kawamura, Y. & Uemura, M. Cryobehavior of the plasma membrane in protoplasts isolated from cold-acclimated *Arabidopsis* leaves is related to surface area regulation. *Plant Cell Physiol.* **49**, 944–957 (2008).
- Zarka, D. G., Vogel, J. T., Cook, D. & Thomashow, M. F. Cold induction of Arabidopsis *CBF* genes involves multiple ICE (inducer of *CBF* expression) promoter elements and a cold-regulatory circuit that is desensitized by low temperature. *Plant Physiol.* **133**, 910–918 (2003).
- Miura, K. & Furumoto, T. Cold signaling and cold response in plants. *Int. J. Mol. Sci.* **14**, 5312–5337 (2013).
- Gilmour, S. J. *et al.* Low temperature regulation of the Arabidopsis CBF family of AP2 transcriptional activators as an early step in cold-induced *COR* gene expression. *Plant J.* **16**, 433–442 (1998).
- Stockinger, E. J., Gilmour, S. J. & Thomashow, M. F. Arabidopsis thaliana CBF1 encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a cis-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. *Proc. Natl. Acad. Sci. USA* **94**, 1035–1040 (1997).
- Liu, Q. *et al.* Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in Arabidopsis. *Plant Cell* **10**, 1391–1406 (1998).

30. Park, S. *et al.* Regulation of the Arabidopsis CBF regulon by a complex low-temperature regulatory network. *Plant J.* **82**, 193–207 (2015).
31. Vogel, J. T., Zarka, D. G., Van Buskirk, H. A., Fowler, S. G. & Thomashow, M. F. Roles of the CBF2 and ZAT12 transcription factors in configuring the low temperature transcriptome of Arabidopsis. *Plant J.* **41**, 195–211 (2005).
32. Zhao, C. *et al.* Mutational evidence for the critical role of CBF transcription factors in cold acclimation in Arabidopsis. *Plant Physiol.* **171**, 2744–2759 (2016).
33. Jia, Y. *et al.* The *cbf3* triple mutants reveal the essential functions of CBFs in cold acclimation and allow the definition of CBF regulons in Arabidopsis. *New Phytol.* **212**, 345–353 (2016).
34. Knight, M. R. & Knight, H. Low-temperature perception leading to gene expression and cold tolerance in higher plants. *New Phytol.* **195**, 737–751 (2012).
35. Sangwan, V., Orvar, B. L., Beyerly, J., Hirt, H. & Dhindsa, R. S. Opposite changes in membrane fluidity mimic cold and heat stress activation of distinct plant MAP kinase pathways. *Plant J.* **31**, 629–638 (2002).
36. Galon, Y., Finkler, A. & Fromm, H. Calcium-regulated transcription in plants. *Mol. Plant.* **3**, 653–669 (2010).
37. Liu, J., Whalley, H. J. & Knight, M. R. Combining modelling and experimental approaches to explain how calcium signatures are decoded by calmodulin-binding transcription activators (CAMTAs) to produce specific gene expression responses. *New Phytol.* **208**, 174–187 (2015).
38. Whalley, H. J. & Knight, M. R. Calcium signatures are decoded by plants to give specific gene responses. *New Phytol.* **197**, 690–693 (2013).
39. Doherty, C. J., Van Buskirk, H. A., Myers, S. J. & Thomashow, M. F. Roles for Arabidopsis CAMTA transcription factors in cold-regulated gene expression and freezing tolerance. *Plant Cell* **21**, 972–984 (2009).
40. Kim, Y., Park, S., Gilmour, S. J. & Thomashow, M. F. Roles of CAMTA transcription factors and salicylic acid in configuring the low-temperature transcriptome and freezing tolerance of Arabidopsis. *Plant J.* **75**, 364–376 (2013).
41. Meyerhoff, O. *et al.* *AtGLR3.4*, a glutamate receptor-like gene is sensitive to touch and cold. *Planta* **222**, 418–427 (2005).
42. Nawaz, Z., Kakar, K. U., Saand, M. A. & Shu, Q. Y. Cyclic nucleotide-gated ion channel gene family in rice, identification, characterization and experimental analysis of expression response to plant hormones, biotic and abiotic stresses. *BMC Genomics* **15**, 853 (2014).
43. Pearcec, R. S. & McDonald, I. Ultrastructural damage due to freezing followed by thawing in shoot meristem and leaf mesophyll cells of tall fescue (*Festuca arundinacea* Schreb.). *Planta* **134**, 159–168 (1977).
44. Miura, K., Ohta, M., Nakazawa, M., Ono, M. & Hasegawa, P. M. ICE1 Ser403 is necessary for protein stabilization and regulation of cold signaling and tolerance. *Plant J.* **67**, 269–279 (2011).
45. Zakhleniuk, O. V., Raines, C. A. & Lloyd, J. C. *pho3*: a phosphorus-deficient mutant of *Arabidopsis thaliana* (L.) Heynh. *Planta*. **212**, 529–534 (2001).
46. Miura, K. & Ohta, M. SIZ1, a small ubiquitin-related modifier ligase, controls cold signaling through regulation of salicylic acid accumulation. *J. Plant Physiol.* **167**, 555–560 (2010).
47. Miura, K. *et al.* SIZ1-mediated sumoylation of ICE1 controls *CBF3/DREB1A* expression and freezing tolerance in Arabidopsis. *Plant Cell* **19**, 1403–1414 (2007).

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Author Contributions

Hidetoshi Iida and Kenji Miura contributed to designing the experiments. Kendo Mori, Na Renhu, Maho Naito, Aki Nakamura, and Hayato Shiba performed the experiments, and collected and analyzed the data. Tsuyoshi Yamamoto, Takuya Suzuki, Hidetoshi Iida, and Kenji Miura contributed to data interpretation and preparation of the manuscript. All authors reviewed the manuscript.

Additional Information

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