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RESEARCH PAPER

A calcium sensor calcineurin B-like 9 negatively regulates cold tolerance via calcium signaling in *Arabidopsis thaliana*

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ABSTRACT

Calcineurin B-like protein 9 (CBL9) plays important roles in response to ABA, K^+ deprivation in plants. However, whether CBL9 modulates plant adaptation to low-temperature stress is elusive. In this study, we demonstrated that the *cbl9* mutants increased freezing tolerance under both cold-acclimating and nonacclimating conditions in *Arabidopsis*. Cold-induced changes of cytosolic free calcium concentration ($[Ca^{2+}]_{cyt}$) were then monitored by aequorin-expressed *Arabidopsis* plants. The results showed that the cold-triggered increases in $[Ca^{2+}]_{cyt}$ levels in *cbl9* mutants were clearly higher than those in wild type (WT) plants, while cold-affected changes in free calcium concentration within cytosolic microdomains adjacent to the vacuolar membrane ($[Ca^{2+}]_{md}$) in *cbl9* mutants were similar to those in WT plants. In addition, treatments of seedlings with Ca^{2+} chelator ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) and Ca^{2+} channel blocker lanthanum chloride markedly inhibit changes of $[Ca^{2+}]_{cyt}$ in *cbl9* mutants, while the inhibition of calcium release by lithium chloride from intracellular pools demonstrated consistent suppression of $[Ca^{2+}]_{cyt}$ in *cbl9* mutants and WT plants. Together, these results indicate that CBL9 negatively modulates cold tolerance through decreasing $[Ca^{2+}]_{cyt}$ in *Arabidopsis*.

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Introduction

Cold stress as an environmental factor restricts the growth and development of plant and remarkably reduces crop quality and productivity. Many temperate plant species can enhance their freezing tolerance after exposure to chilling temperature, the adaptive process known as cold acclimation.[1] Complex physiological changes are involved in cold acclimation including enhancement stability of membranes and production of cryoprotective proteins and low-molecular-weight cryoprotectants.[2] A set of marker genes are induced in cold acclimation. These genes include *KIN* (cold inducible), *COR* (cold regulated), *RD* (responsive to dehydration), and *LTI* (low temperature induced).[3] Some proteins like enzymes, antifreeze polypeptide, and molecular chaperones can also increase tolerance to the dehydration caused by low temperature in plants.[4,5]

In plant cells, calcium functions as a second messenger in a wide range of signal transduction networks.[6] It has been demonstrated that the earliest response to low temperature is a transient increase in $[Ca^{2+}]_{cyt}$. $[Ca^{2+}]_{cyt}$ rise has been shown to be initiated by calcium influx through the plasma membrane from the extracellular calcium stores and by calcium release from intracellular calcium stores.[7] In addition, electrophysiology study has shown that mechanosensitive Ca^{2+} channels are regulated by temperature in *Arabidopsis* mesophyll cells.[8] The more important evidence of calcium behaves as a second messenger in low-temperature signaling is the prevention of cold acclimation by Ca^{2+} channel blockers and Ca^{2+} chelators.[9] Thus, Ca^{2+} acts as a second messenger in response to low-temperature stress and cold acclimation.[8]

Calcium sensors unscramble the temporal and spatial changes of Ca^{2+} concentrations in calcium signaling molecular pathways.[10] As calcium sensor protein, calcineurin B-like proteins (CBL) in plants are similar to calcineurin B (CNB) and neuronal calcium sensors from animals.[11] CBL proteins containing EF-hand domains for calcium binding specifically interact with a set of serine–threonine protein kinases named as CBL-interacting protein kinases (CIPKs).[12] Many types of research have confirmed a wide range of key functions of the CBL–CIPK network to cope with the environmental changes in plants. Different CBL–CIPK combination pairs appear to participate in specific signal transduction pathways and may have functional overlap. To date, at least ten *Arabidopsis* CBLs have been identified. The *Arabidopsis* CBLs share 20–90% amino acid sequence identity. It is supposed that CBL proteins would have high functional redundancy among closely related members while supporting functional specificity among highly divergent members.[13] For example, *SOS3* (*CBL4*) encodes a calcium sensor and is specifically involved in plant salt tolerance stress, whereas *CBL1* has a positive role in regulating salt and drought stress and a negative role in regulating cold stress in *Arabidopsis*.[14]

Previous studies have shown that *CBL9* negatively regulates ABA and osmotic stress responses and is involved in the absorption of potassium under low potassium conditions in plants.[15,16] *Arabidopsis CBL* proteins are also responsible for numerous regulation of other stress in different plant signal

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transduction processes. Despite being involved in multiple stress responses, little is known about the function of CBL9 in responding to low temperatures in plants. Here, we reported that CBL9 decreased freezing tolerance through the depression of transient increase of $[Ca^{2+}]_{cyt}$ induced by cold stress in *Arabidopsis*.

Results

CBL9 mutant was insensitive to freezing

To define the function of *CBL9* in response to cold stress, we identified Salk_142774 that contained T-DNA insertion in the *CBL9* gene.[16] The site of T-DNA insertion is located in the promoter of *CBL9* (Figure 1a) and was approved by PCR using the *CBL9*-specific primer and the T-DNA left border primer. RT-PCR results revealed that *CBL9* was disrupted in the *cbl9* mutant (Figure 1b). Under normal conditions, the growth and development of *cbl9* mutants were not significantly different from WT plants (Figure 1c, d). However, the *cbl9* mutants displayed freezing-insensitive phenotype compared with WT plants under both cold-acclimating and nonacclimating conditions (Figure 1c, d). Under nonacclimating conditions, 29.5 ± 8.26% of the WT plants survived the freezing test compared to 55.8 ± 10.38% of the *cbl9* mutants (Figure 1e). Approximately 44.5 ± 8.13% of WT plants and 89.5 ± 7.52% of *cbl9* mutants recovered from the freezing treatment after cold-acclimating (Figure 1f). These results indicate that *cbl9* mutants are less sensitive to freezing stress than WT plants.

Cold-induced increases in intracellular Ca^{2+} levels were promoted in *cbl9*

Previous data indicated that cold shock induced a transient calcium increase, which then triggered downstream responses.[7] Thus, the freezing-insensitive phenotypes of *cbl9* might be due to changes in calcium signature. To monitor cold stress-induced $[Ca^{2+}]_{cyt}$ responses, we generated transgenic *Arabidopsis* plants stably transformed with the plasmid of *MAQ2.4*.[17] As shown in Figure 2a, *cbl9* mutants exhibited a higher peak of $[Ca^{2+}]_{cyt}$ (1.96 ± 0.078 μM) than the WT plants (1.66 ± 0.057 μM). Statistical analysis showed that *cbl9's* $[Ca^{2+}]_{cyt}$ peak was significantly different from WT plants in response to cold shock (Figure 2b). Cold shock $[Ca^{2+}]_{cyt}$ responses involve both a transmembrane influx of external calcium and calcium

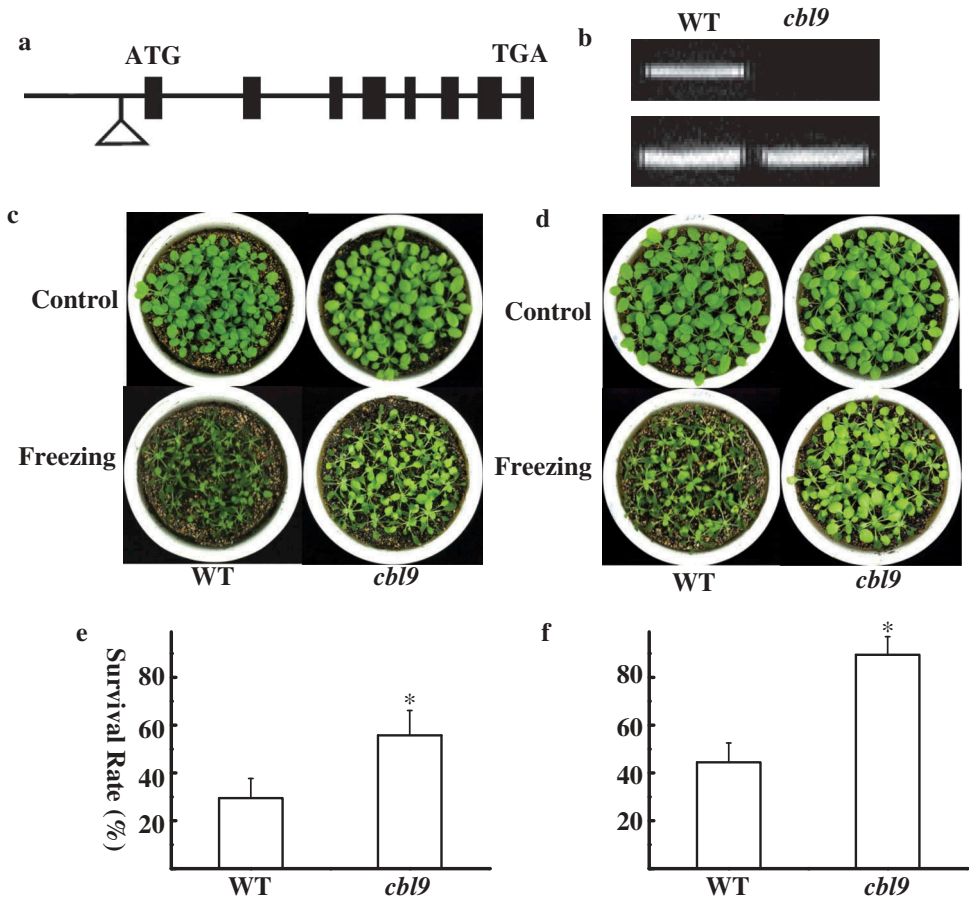

Figure 1. Mutation of *CBL9* leads to stronger freezing tolerance in *Arabidopsis thaliana*.

(a) Insertion position of T-DNA in the *CBL9* gene. Filled black boxes represent exons, lines represent introns, and the triangle represents T-DNA insertion. (b) RT-PCR analysis of *CBL9* transcript levels. *Actin2* was as a loading control. (c-d) Freezing phenotypes. Three-week-old seedlings grown in soil were subjected to the freezing assay. For nonacclimated treatment, the seedlings were directly subjected at −6°C for 8 h (c). For acclimated treatment (pretreated at 4°C for 4 d), the seedlings were subjected at −6°C for 8 h (d). The pictures were taken 7 d after treatments. The phenotype of seedlings before (upper) and after (bottom) freezing treatment was shown. (e-f) Survival rates of WT (left) and *cbl9* (right) in (c-d). The data are the mean values of three replicates ± SD (n = 120). Asterisk indicates a significant difference ($P < 0.05$, t test) between WT and *cbl9* mutants. Similar results were observed in three independent experiments.

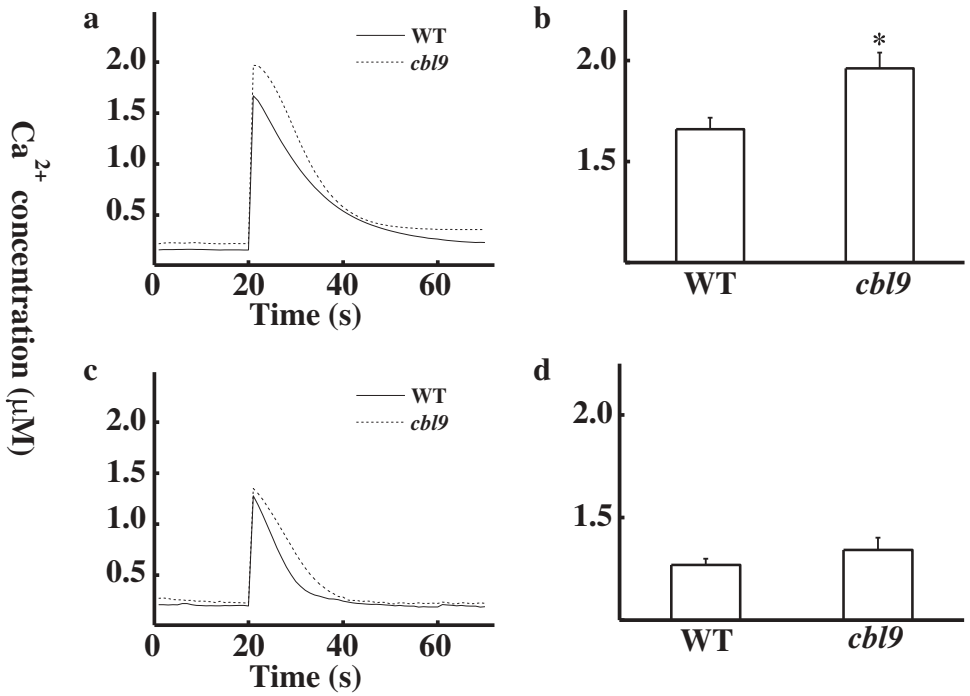

Figure 2. Kinetics of the cold-induced elevation of the $[Ca^{2+}]$ in WT and *cbl9* mutants.
(a) Elevation of $[Ca^{2+}]_{cyt}$ in response to cold shock in WT and *cbl9* mutants. 7 d old seedlings expressing cytosolic aequorin were subjected to cold shock. A representative trace is shown. (b) The averages of $[Ca^{2+}]_{cyt}$ at the peaks (WT, n = 7; *cbl9*, n = 8) were shown. Asterisk indicates a significant difference ($P < 0.05$, *t* test) between WT and *cbl9* mutants. (c) Elevation of $[Ca^{2+}]_{md}$ in response to cold shock in WT and *cbl9* mutants. Seven-d-old seedlings expressing microdomain aequorin were subjected to cold shock. A representative trace is shown. (d) The averages of $[Ca^{2+}]_{md}$ at the peaks (WT, $n = 10$; *cbl9*, $n = 11$) were shown.

signaling events on the tonoplast.[7] To investigate the source of intracellular calcium transient in response to cold, *Arabidopsis* was transformed with the plasmid of *HVA1*. *HVA1* encodes a proton pyrophosphatase–apoaequorin fusion protein that expresses aequorin in the cytosolic microdomain adjacent to the vacuolar membrane.[7] As shown in Figure 2c, after the cold shock, the $[Ca^{2+}]_{md}$ peak of WT and *cbl9* mutants achieved similar levels, with average peak height of 1.27 ± 0.03 μM and 1.34 ± 0.06 μM, respectively. Statistical analysis showed that $[Ca^{2+}]_{md}$ peak in *cbl9* mutants in response to cold shock was not significantly different from WT plants (Figure 2d). These data suggest at least in part contribution of calcium influx from extracellular but not from intracellular space induced by cold shock in *cbl9* mutants that displayed enhanced freezing tolerance.

Effects of various inhibitors on cold shock-induced $[Ca^{2+}]_{cyt}$ transients

To further ascertain the involvement of extracellular or intracellular calcium in cold $[Ca^{2+}]_{cyt}$ response in *cbl9* mutants, the transformed *MAQ2.4 Arabidopsis* seedlings were pretreated with several calcium-signaling inhibitors. After reconstitution of aequorin, inhibitors were added for incubation as indicated in the figures. When plants were treated with Ca^{2+} antagonist $LaCl_3$, both WT and *cbl9* mutants exhibited reduced cold-induced increase in $[Ca^{2+}]_{cyt}$ (Figure 3a). As shown in Figure 3b, the peak height of WT and *cbl9* mutants was decreased from 1.69 ± 0.08 μM to 0.92 ± 0.05 μM and from 1.90 ± 0.07 μM to 0.79 ± 0.09 μM, respectively, and cold shock-induced $[Ca^{2+}]_{cyt}$ increase tends to be consistent in WT and *cbl9* mutants. Chelation of extracellular calcium by EGTA showed similar results (Figure 3c). The peak height of WT and *cbl9* mutants was decreased from 1.67 ± 0.08 μM to 1.23 ± 0.11 μM and from 1.98 ± 0.01 μM to 1.18 ± 0.05 μM, respectively, and cold shock-induced $[Ca^{2+}]_{cyt}$ increase also tends to be consistent (Figure 3d). The effect of EGTA and $LaCl_3$ suggests that extracellular Ca^{2+} is at least, in part, an important source for the $[Ca^{2+}]_{cyt}$ increase in the WT and *cbl9* mutants under cold stress, most likely due to lower greater scope of *cbl9* $[Ca^{2+}]_{cyt}$. IP_3 was involved in a cold shock-induced calcium release from intracellular calcium pools. As a potent inhibitor of the phosphatidylinositol cycle, LiCl inhibits IP3 signaling and prevents calcium release from intracellular pools.[7] Plants were pretreated with LiCl to determine whether the different changes of cold-induced $[Ca^{2+}]_{cyt}$ occurred from intracellular calcium pools. As shown in Figure 4a, LiCl led to a reduction in the peak of the Ca^{2+} concentration in response to cold shock. Moreover, the decreased extent of WT plants was notably different from that of *cbl9* mutants. $[Ca^{2+}]_{cyt}$ of WT peaked at 1.37 ± 0.04 μM compared with 1.71 ± 0.08 μM for the control, while $[Ca^{2+}]_{cyt}$ of *cbl9* peaked at 1.81 ± 0.05 μM compared with 1.98 ± 0.03 μM for the control. The *cbl9* mutants also exhibited a higher peak than the WT did (Figure 4b). The fact that the peak of *cbl9* mutants was affected to a greater extent by $LaCl_3$ and EGTA inhibition than by IP3 signaling inhibition suggested that the higher $[Ca^{2+}]_{cyt}$ elevation in *cbl9* mutants mediated by cold shock suggested a significant contribution of extracellular calcium pools.

Discussion

The geographical location of temperate and cultivated plants is limited by low temperatures, and freezing temperatures

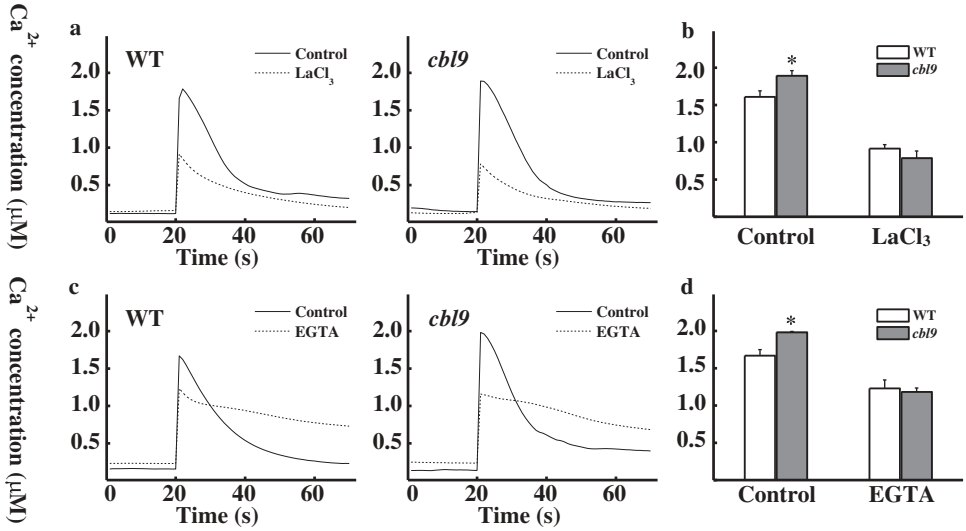

Figure 3. Effect of Ca^{2+} channel blocker $LaCl_3$ and Ca^{2+} chelator EGTA on cold shock-induced $[Ca^{2+}]_{cyt}$ transients in WT and *cbl9* mutants.
(a) Effect of $LaCl_3$ pretreatment for 1 h on cold shock-induced $[Ca^{2+}]_{cyt}$ changes in WT and *cbl9* mutants. A representative trace is shown. (b) The averages of $[Ca^{2+}]_{cyt}$ at the peaks (WT, $n = 6$; *cbl9*, $n = 6$) in (a) were shown. (c) Effect of EGTA pretreatment for 1 h on cold shock-induced $[Ca^{2+}]_{cyt}$ changes in WT and *cbl9* mutants. A representative trace is shown. (d) The averages of $[Ca^{2+}]_{cyt}$ at the peaks (WT, $n = 9$; *cbl9*, $n = 10$) in (c) were shown. In (b) and (d), asterisks indicate a significant difference ($P < 0.05$, *t* test) between WT and *cbl9* mutants.

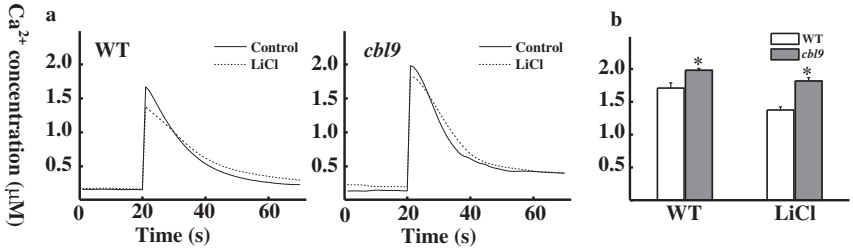

Figure 4. Effect of inhibiting IP3 metabolism on cold shock-induced $[Ca^{2+}]_{cyt}$ transients in WT and *cbl9* mutants.
(a) Effect of LiCl pretreatment for 30 min on cold shock-induced $[Ca^{2+}]_{cyt}$ changes in WT and *cbl9* mutants. A representative trace is shown. (b) The averages of $[Ca^{2+}]_{cyt}$ at the peaks (WT, $n = 8$; *cbl9*, $n = 8$) in (a) were shown. Asterisks indicate a significant difference ($P < 0.05$, *t* test) between WT and *cbl9* mutants.

may significantly reduce crop production.[1] To improve the freezing resistance of cultivated crops, traditional plant breeding approaches encounter many problems. Uncovering the molecular mechanisms that confer plant tolerance to freezing would not only improve the fundamental knowledge of how plants adapt to environmental changes but could have implications for the development of new strategies to increase the freezing tolerance of crop species.[1,14] Previous evidence indicates that *CBL9* is a negative regulator of ABA responses and osmotic stress, and its normal function desensitizes ABA effects on seed germination and seedling growth by reducing ABA-induced gene expression.[15] Meanwhile, CBL9 and CBL1 bind to and activate protein kinase CIPK23 that directly phosphorylates the K^+ transporter AKT1 and enhance K^+ uptake in *Arabidopsis* roots.[16] Here, our study provides evidence that CBL9 plays a negative role in freezing tolerance by decreasing $[Ca^{2+}]_{cyt}$. It was shown that the *cbl9* mutants render plants insensitive to cold stress, thus supporting the importance of calcium sensor for adapting to cold stress.

CBL-type calcium sensors belong to small EF-hand proteins that constitute a distinctive family of calcium sensors. CBLs sense the calcium signals generated by a wide large of stresses and then transmit information to different kinds of protein kinases named CIPKs. It is suggested that Ca^{2+} sensors and their target protein are involved in decoding calcium signaling 'specificity'. Ca^{2+} signature was decoded by various signal transduction pathways.[18] For example, CBL1 positively regulates salt and drought responses and negatively regulates cold response, and CBL4/SOS3 modulates salt tolerance in plants.[19] By contrast, CBL10 is involved in salt tolerance pathway by regulating AKT1 activity to affect ion homeostasis.[20] Our studies have demonstrated that *cbl9* mutants have increased tolerance to freezing stress under both cold-acclimating and nonacclimating conditions (Figure 1).

Ca^{2+} is an effective protecting agent, and so a $[Ca^{2+}]_{cyt}$ rise is generally benefit for cold stress conditions. Ca^{2+} generally ameliorates stress injury on the cellular level.[21] The plasma membrane is considered to be the primary site of injury when plants are subjected to cold stress.[1] In addition, it is observed that Ca^{2+} tightens membranes, decreases passive ion fluxes, and renders membranes more hydrophobic. This suggests that Ca^{2+} has a universal role in membrane stability and cell integrity for increasing stress resistance.[21] In contrast, the disaccumulation of membrane-

associated Ca^{2+} can cause damage to membrane stability, and Ca^{2+} may protect the membrane from freezing stress.[22] Maybe the different changes of Ca^{2+} concentration are the reason why the *cbl9* mutants are more insensitive to freezing stress than WT.

It has been shown that the oscillation frequency of $[Ca^{2+}]_{cyt}$ transmits the efficiency and specificity of cellular responses.[23] It seems that subtle differences in the Ca^{2+} signatures are a requirement for eliciting physiological responses. Oxidative stress and extracellular Ca^{2+} elicited prolonged Ca^{2+} increases without oscillation in *Arabidopsis* V-ATPase mutant *de-etiolated 3* (*det3*), whereas WT cells show $[Ca^{2+}]_{cyt}$ oscillations. These data indicate that stimulation-induced specific Ca^{2+} oscillations are essential for long-term stomatal closure.[24] However, there are little data on how these calcium sensors might decode calcium transients. As calcium-binding proteins, the CBL proteins are the most important relay for calcium signaling in plant. Thus, the CBL mutants provided a useful tool to investigate the regulation of calcium signaling. Cold-triggered calcium responses in *cbl9* mutants were higher than those in WT in $[Ca^{2+}]_{cyt}$ (Figure 2b) but were similar in $[Ca^{2+}]_{md}$ (Figure 2d). Pharmacological studies have shown that such difference was mainly related to extracellular calcium transmembrane transport (Figures 3 and 4). CBL9 coupled with certain CIPK may mediate downstream signaling responses or feedback regulation of Ca^{2+} gradient during cold stress. As Ca^{2+} sensor relaying system, the CBL–CIPK network maintained Ca^{2+} homeostasis by a feedback mechanism. *Arabidopsis* CIPK19 was involved in regulating Ca^{2+} influx by mediating plasma membrane Ca^{2+} channels at the tip of the pollen tube.[25] We speculated that CBL9 with certain CIPK act as a feedback mechanism to reduce the Ca^{2+} signaling strength when cytoplasmic Ca^{2+} concentration reaches a higher state level. Ca^{2+} networks with amplifying pathways and feedback loops induced Ca^{2+} oscillation.[26] The downregulated Ca^{2+} transients by CBL9 may trigger Ca^{2+} oscillations. This hypothesis can also explain that the animal ER-type calcium pumps were regulated by a feedback system that maintains proper Ca^{2+} load and prevents cytotoxic Ca^{2+} overload.[27] In the future, identifying CIPKs that interact with CBL9 will provide further insight into CBL9-mediated pathway regulating Ca^{2+} homeostasis in cold stress.

As a calcium sensor, CBL9 negatively modulates freezing tolerance in *Arabidopsis*. Although further evidence should be sought to uncover the participation of other calcium sensors in low-temperature signaling, this study reveals new insights into the molecular mechanism of response to low-temperature stress and could enable development of novel ways to enhance freezing tolerance of crop plants.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia-0 (Col-0) was used as the WT plants in this study. Plants were grown at 22 ± 2°C under a 16-h-light/8-h-dark photoperiod at 100 μmol m^{-2} s^{-1} with 70% relative humidity in soil or on 1/2 Murashige and Skoog (MS) medium (Sigma) containing 0.8% agar and 1.5% sucrose.

Identification and isolation of *cbl9* mutants

T-DNA insertion line of *CBL9* (At5g47100), Salk_142774 was received from the *Arabidopsis* Biological Resource Center. The homozygous nature of the mutant was confirmed by PCR amplification according to http://signal.salk.edu/tdnaprimers.2.html. A recessive mutation of a single T-DNA insertion locus was confirmed as our laboratory previously described and approved from other studies .[16,28]

Freezing assay

Freezing tolerance assays were performed as described previously,[29] with minor modifications. Three-week-old seedlings grown in soil were subjected to the freezing assay. For nonacclimated treatment, the seedlings were directly subjected to the freezing assay. For acclimated treatment, the seedlings were first grown at 4°C for 4 d under a 16-h-light/8-h-dark photoperiod and then subjected to the freezing assay. The freezing assays for both nonacclimated and acclimated seedlings were subjected at −6°C for 8 h. After treatment, the seedlings were transferred at 4°C for 12 h in the dark and then transferred to a growth chamber for another 7 d. After recovery, seedlings that could still grow new leaves were counted as survivors, and the survival rates were measured.

$[Ca^{2+}]_{cyt}$ measurements in cbl9 and the WT plants

The plasmids of *MAQ2.4* and *HVA1* were transferred into *Agrobacterium tumefaciens* strain GV3101 and transformed, respectively, into WT and *cbl9* mutants by floral infiltration. Transgenic *Arabidopsis* plants expressing *MAQ2.4* were used for $[Ca^{2+}]_{cyt}$ measurements. Plants expressing *HVA1* were used for $[Ca^{2+}]_{md}$ measurement. Aequorin was reconstituted *in vivo* by incubating 7–8 d *Arabidopsis* seedlings on water containing 2.5 μM native coelenterazine (Promega, WI, USA) for 12 h in the dark at 25°C ± 2°C. Cold shock-induced cytosolic Ca^{2+} concentration changes were measured according to Ref. 7. Luminescence counts were recorded by a digital luminometer (TD20/20^n, Turner Biosystems, CA, USA), and luminescence values were converted into actual Ca^{2+} concentrations as described previously.[7] Inhibitor experiments were performed by placing reconstituted seedlings in 10 mM $LaCl_3$ for 1 h, in 20 mM EGTA for 1 h, and in 20 mM LiCl for 30 min (all Sigma, USA). After which, seedlings were removed from the inhibitor solution and put in luminometer for calcium concentration measurement as given above.

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Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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References

1. Thomashow MF. Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. Annu Rev Plant Physiol Plant Mol Biol. 1999;50:571–599. doi:10.1146/annurev.arplant.50.1.571.
2. Zhao C, Zhang Z, Xie S, Si T, Li Y, Zhu JK. Mutational evidence for the critical role of CBF transcription factors in cold acclimation in *Arabidopsis*. Plant Physiol. 2016;171:2744–2759. doi:10.1104/pp.16.00533.
3. Li H, Ding Y, Shi Y, Zhang X, Zhang S, Gong Z, Yang S. MPK3- and MPK6-mediated ICE1 phosphorylation negatively regulates ICE1 stability and freezing tolerance in *Arabidopsis*. Dev Cell. 2017;43:630–642. doi:10.1016/j.devcel.2017.09.025.
4. Janmohammadi M, Zolla L, Rinalducci S. Low temperature tolerance in plants: changes at the protein level. Phytochemistry. 2015;117:76–89. doi:10.1016/j.phytochem.2015.06.003.
5. Jha UC, Bohra A, Jha R. Breeding approaches and genomics technologies to increase crop yield under low-temperature stress. Plant Cell Rep. 2017;36:1–35. doi:10.1007/s00299-016-2073-0.
6. Raffaello A, Mammucari C, Gherardi G, Rizzuto R. Calcium at the center of cell signaling: interplay between endoplasmic reticulum, mitochondria, and lysosomes. Trends Biochem Sci. 2016;41:1035–1049. doi:10.1016/j.tibs.2016.09.001.
7. Knight H, Trewavas AJ, Knight MR. Cold calcium signaling in *Arabidopsis* involves two cellular pools and a change in calcium signature after acclimation. Plant Cell. 1996;8:489–503. doi:10.1105/tpc.8.3.489.
8. Mori K, Renhu N, Naito M, Nakamura A, Shiba H, Yamamoto T, Suzaki T, Iida H, Miura K. Ca^{2+}-permeable mechanosensitive channels MCA1 and MCA2 mediate cold-induced cytosolic Ca^{2+} increase and cold tolerance in *Arabidopsis*. Sci Rep. 2018;8:550. doi:10.1038/s41598-017-17483-y.
9. Monroy AF, Sarhan F, Dhindsa RS. Cold-induced changes in freezing tolerance, protein phosphorylation, and gene expression (evidence for a role of calcium). Plant Physiol. 1993;102:1227–1235. doi:10.1104/pp.102.4.1227.
10. Ranty B, Aldon D, Cotelle V, Galaud JP, Thuleau P, Mazars C. Calcium sensors as key hubs in plant responses to biotic and abiotic stresses. Front Plant Sci. 2016;7:327. doi:10.3389/fpls.2016.00327.
11. Kudla J, Xu Q, Harter K, Gruissem W, Luan S. Genes encoding calcineurin B-like protein in *Arabidopsis* are differentially regulated by stress signals. Proc Natl Acad Sci USA. 1999;96:4718–4723. doi:10.1073/pnas.96.8.4718.
12. Yu Q, An L, Li W. The CBL-CIPK network mediates different signaling pathways in plants. Plant Cell Rep. 2014;33:203–214. doi:10.1007/s00299-013-1507-1.
13. Kolukisaoglu U, Weinl S, Blazevic D, Batistic O, Kudla J. Calcium sensors and their interacting protein kinases: genomics of the *Arabidopsis* and rice CBL-CIPK signaling networks. Plant Physiol. 2004;134:43–58. doi:10.1104/pp.103.033068.
14. Zhu JK. Abiotic stress signaling and responses in plants. Cell. 2016;167:313–324. doi:10.1016/j.cell.2016.08.029.
15. Pandey GK, Cheong YH, Kim KN, Grant JJ, Li L, Hung W, D'Angelo C, Weinl S, Kudla J, Luan S. The calcium sensor calcineurin B-like 9 modulates abscisic acid sensitivity and biosynthesis in *Arabidopsis*. Plant Cell. 2004;16:1912–1924. doi:10.1105/tpc.021311.
16. Xu J, Li HD, Chen LQ, Wang Y, Liu LL, He L, Wu WH. A protein kinase, interacting with two calcineurin B-like proteins, regulates K^+ transporters AKT1 in *Arabidopsis*. Cell. 2006;125:1347–1360. doi:10.1016/j.cell.2006.06.011.
17. Knight MR, Campbell AK, Smith SM, Trewavas AJ. Transgenic plant aequorin reports the effects of touch and cold-shock and elicitors on cytoplasmic calcium. Nature. 1991;352:524–526. doi:10.1038/352524a0.
18. Luan S. The CBL-CIPK network in plant calcium signaling. Trends Plant Sci. 2009;14:37–42. doi:10.1016/j.tplants.2008.10.005.
19. Cheong YH, Kim KN, Pandey GK, Gupta R, Grant JJ, Luan S. CBL1, a calcium sensor that differentially regulates salt, drought, and cold responses in *Arabidopsis*. Plant Cell. 2003;15:1833–1845. doi:10.1105/tpc.012393.
20. Monihan SM, Magness CA, Yadegari R, Smith SE, Schumaker KS. *Arabidopsis* CALCINEURIN B-LIKE10 functions independently of the SOS pathway during reproductive development in saline conditions. Plant Physiol. 2016;171:369–379. doi:10.1104/pp.16.00334.
21. Plieth C. Plant calcium signaling and monitoring: pros and cons and recent experimental approaches. Protoplasma. 2001;218:1–23. doi:10.1007/BF01288356.
22. Hirschi KD. Expression of *Arabidopsis CAX1* in tobacco: altered calcium homeostasis and increased stress sensitivity. Plant Cell. 1999;11:2113–2122. doi:10.1105/tpc.11.11.2113.
23. Sneyd J, Han JM, Wang L, Chen J, Yang X, Tanimura A, Sanderson MJ, Kirk V, Yule DI. On the dynamical structure of calcium oscillations. Proc Natl Acad Sci USA. 2017;114:1456–1461. doi:10.1073/pnas.1614613114.
24. Allen GJ, Chu SP, Schumacher K, Shimazaki CT, Vafeados D, Kemper A, Hawke SD, Tallman G, Tsien RY, Harper JF, et al. Alteration of stimulus-specific guard cell calcium oscillations and stomatal closing in *Arabidopsis det3* mutant. Science. 2000;289:2338–2342. doi:10.1126/science.289.5488.2338.
25. Zhou L, Lan W, Chen B, Fang W, Luan S. A calcium sensor-regulated protein kinase, CALCINEURIN B-LIKE PROTEIN-INTERACTING PROTEIN KINASE19, is required for pollen tube growth and polarity. Plant Physiol. 2015;167(4):1351–1360. doi:10.1104/pp.114.256065.
26. Plieth C. Calcium: just another regulator in the machinery of life? Ann Bot. 2005;96:1–8. doi:10.1093/aob/mci144.
27. Bhogal MS, Colyer J. Depletion of Ca^{2+} from the sarcoplasmic reticulum of cardiac muscle prompts phosphorylation of phospholamban to stimulate store refilling. Proc Natl Acad Sci USA. 1998;95:1484–1489. doi:10.1073/pnas.95.4.1484.
28. Bai L, Zhang G, Zhou Y, Zhang Z, Wang W, Du Y, Wu Z, Song CP. Plasma membrane-associated proline-rich extensin-like receptor kinase 4, a novel regulator of Ca^{2+} signalling, is required for abscisic acid responses in *Arabidopsis thaliana*. Plant J. 2009;60:314–327. doi:10.1111/j.1365-313X.2009.03956.x.
29. Ji H, Wang Y, Cloix C, Li K, Jenkins GI, Wang S, Shang Z, Shi Y, Yang S, Li X. The *Arabidopsis* RCC1 family protein TCF1 regulates freezing tolerance and cold acclimation through modulating lignin biosynthesis. PLoS Genet. 2015;11:e1005471. doi:10.1371/journal.pgen.1005471.