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Membrane rigidification functions upstream of the MEKK1-MKK2-MPK4 cascade during cold acclimation in *Arabidopsis thaliana*

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ABSTRACT

The MEKK1-MKK2-MPK4 cascade is activated during cold acclimation. However, little is known regarding the perception of low temperature. In this study, we demonstrate that treatment of *Arabidopsis* with a membrane rigidifier, DMSO, caused MPK4 activation concomitantly with MEKK1 and MKK2 phosphorylation, as well as the cold-inducible gene *COR15a* expression. These processes are similar to the effects of cold treatment, whereas benzyl alcohol (BA), a membrane fluidizer, prevented such cold-induced events. Moreover, the DMSO-treated seedlings acquired freezing tolerance without cold acclimation. In contrast, the BA-pretreated seedlings did not show freezing tolerance. These results suggest that membrane rigidification activates this MAPK cascade and contributes to the acquisition of freezing tolerance.

1. Introduction

Many plants originating from temperate regions, including *Arabidopsis thaliana* L., acquire freezing tolerance through cold acclimation, that is, exposure to low but non-freezing temperatures. Various intracellular signaling pathways involved in cold acclimation have been reported [1,2]. It is also known that mitogen-activated protein kinase (MAPK) cascades play an important role in cold acclimation signaling [3,4]. MAPK cascades are components of the intracellular signaling network and are widely conserved in eukaryotic cells [5–7]. Generally, MAPK is activated by MAPK kinase (MAPKK), which itself is activated by MAPKK kinase (MAPKKK). It has been reported that of the *Arabidospsis* MAPKs, MPK4 and MPK6 are activated during cold signaling [8]. It has also been reported that a MAPKK, MKK2, is activated during cold signaling and phosphorylates MPK4 and MPK6 [9]. We have previously shown that a MAPKKK, MEKK1, phosphorylates MKK2 during cold treatment [10]. In addition, the phosphorylation of MEKK1 via Ca^{2+} signaling has been detected during the cold response [10]. Moreover, the Ca^{2+}/calmodulin (CaM)-regulated receptor-like kinase, CRLK1, a positive regulator of the response to freezing temperatures that is activated in the presence of Ca^{2+}/CaM, can phosphorylate MEKK1 [10–12]. These reports suggest that the Ca^{2+}/CaM-CRLK1–MEKK1-MKK2-MPK4/MPK6 pathway is activated during the cold response. However, little is known about the mechanism of the perception of a temperature downshift upstream of this signaling pathway.

In a variety of organisms such as plants, fishes, bacteria, and cyanobacteria, it has been demonstrated that low temperatures cause a decrease in membrane fluidity (that is, they cause membrane rigidification) [1,13]. In the cyanobacteria system, it has been reported that the temperature downshift might be recognized by a sensor that detects a change in membrane fluidity [14]. It has also been reported that membrane rigidification induces the activation of MAPK in a higher plant, alfalfa [15,16].

To investigate whether a change in membrane fluidity affects cold signaling in *Arabidopsis*, seedlings were treated with either a membrane rigidifer, DMSO, or a membrane fluidizer, benzyl alcohol (BA). MPK4 was activated by DMSO without cold treatment, whereas cold-induced MPK4 activation was suppressed by the BA pretreatment. Phosphorylation of MKK2 and MEKK1, the upstream components of MPK4 in the cold signaling pathway, was also induced by DMSO treatments and decreased by BA pretreatments. These data indicate that membrane rigidification occurs upstream of the MEKK1-MKK2-MPK4 cascade during cold acclimation. The cold-inducible gene, *COR15a,* was expressed by DMSO without cold treatment, whereas its expression was suppressed by the BA pretreatment. Moreover, we determined that the DMSO-treated seedlings acquired freezing tolerance without cold acclimation, and the

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BA-pretreated seedlings did not show freezing tolerance. These results suggest that the membrane ridigification activates this MAPK cascade and contributes to the acquisition of freezing tolerance.

2. Materials and methods

2.1. Plant materials, cold and/or chemical treatments and protein extraction

A. thaliana (Columbia ecotype) seedlings were grown on Gamborg's B5 agar plates at 22 °C under continuous light conditions. Two-week-old seedlings were removed from the agar plate and allowed to stand in water for 24 h before each treatment. Cold treatments were performed by shifting seedlings to 4 °C for the indicated times. For dimethylsulfoxide (DMSO) treatments, the seedlings were soaked in 3% or 6% DMSO solutions at 22 °C for the indicated times. To analyze the effect of benzyl alcohol (BA) treatment on cold signaling, seedlings were placed in 20 mM or 40 mM BA solutions at 22 °C for 1 h, then shifted to 4 °C or kept at 22 °C for the indicated times. These seedlings were ground in liquid nitrogen and then thawed in the extraction buffer (100 mM Tris–HCl (pH 8.0), 1 mM EDTA, 1% Triton X-100, 150 mM NaCl, 1 mM PMSF, 1 μg/ml leupeptin, 2 mM DTT, 1 mM sodium vanadate, 25 mM sodium fluoride and 50 mM β-glycerophosphate). After centrifugation, the supernatants were used for immunoprecipitation, kinase assays and immunoblot analysis.

2.2. Molecular cloning

The MEKK1 (At4g08500), MKK1 (At4g26070) and MKK2 (At4g29810) genes of *A. thaliana* Columbia-0 were obtained by RT-PCR, and the kinase negative mutations of MEKK1 (K361R), MKK1 (K97R) and MKK2 (K99R), designated as MEKK1 KN, MKK1 KN and MKK2 KN, respectively, were created as described previously [10].

2.3. Expression and purification of recombinant proteins

MEKK1 KN, MKK1 KN and MKK2 KN were cloned into a pGEX4T-1 vector (GE Healthcare) for translational fusion to glutathione S-transferase (GST). Each vector was transformed into *Escherichia coli* strain JM109. The recombinant GST-fusion proteins were purified as described previously [17].

2.4. Immunoprecipitation of Arabidopsis MPK4

Crude extracts of *Arabidopsis* (0.5 mg protein) were incubated with a 1000-fold diluted anti-MPK4 antibody solution (Sigma) in a final volume of 1 ml at 4 °C for 1 h, followed by the addition of 20 μl of Protein A Sepharose (GE Healthcare). The mixture was further incubated at 4 °C for 2 h. The collected resin was washed three times with the ice-cold extraction buffer and subjected to kinase assays.

2.5. Kinase assays

Immunoprecipitated MPK4 from each treated seedling was incubated with myelin basic protein (MBP) in a kinase reaction mixture containing 30 mM Tris–HCl (pH 7.5), 100 mM NaCl, 20 mM $MgCl_2$, 50 μM ATP and [γ-^{32}P] ATP (37 kBq) at 30 °C for 20 min. The reaction mixtures were separated by SDS–PAGE on a 15% gel, and the phosphorylation of MBP was visualized with a Bioimaging Analyzer BAS2500 (Fuji). The protein extracts (0.1 μg protein) from each treated seedling were allowed to react with the recombinant GST-fusion MEKK1 KN, MKK1 KN and MKK2 KN proteins in the kinase reaction mixture. The samples were separated by SDS–PAGE on a 10% gel and detected as described above.

2.6. Immunoblot analysis

Immunoblot analysis was performed as described previously [17]. Crude extracts of *Arabidopsis* (20 μg protein) were separated by SDS–PAGE on a 10% gel. After SDS–PAGE, proteins were electroblotted onto an Immobilon P membrane (Millipore, Boston, MA). Anti-MPK4 antibody, diluted 1000-fold, was used as the primary antibody. After extensive washing of the membrane with TBS-T buffer, an alkaline phosphatase-conjugated anti-rabbit second antibody (Promega, Madison, WI) was applied, and the color reaction was performed using 5-bromo-4 chloro-3-indolyl-phosphate and nitro-blue tetrazolium as substrates.

2.7. RT-PCR

Total RNA was extracted from the cold, DMSO, and BA *treated seedlings* using an RNeasy Plant Mini Kit (Qiagen). The cDNA was synthesised from 0.5 μg of total RNA using a PrimeScript 1st strand cDNA Synthesis Kit (TAKARA). *COR15a* (At2g42540) transcripts were amplified using the forward primer, 5′-GCT TCA GAT TTC GTG ACG GAT AAA AC-3′, and the reverse primer, 5′-GCA AAA CAT TAA AGA ATG TGA CGG TG-3′. The PCR was performed using Hot Start *Taq* DNA Polymerase (New England Biolabs) at 95 °C for 2 min, followed by 24 cycles at 95 °C for 30 s, 50 °C for 30 s and 68 °C for 45 s, with a final extension at 68 °C for 5 min. *Actin8* (At1g49420) transcripts were used as a control and were amplified using the forward primer, 5′-GAA GGA CCT TTA CGG TAA CA-3′, and the reverse primer, 5′-CCA ATC CAG ACA CTG TAC TT-3′. The PCR was performed at 95 °C for 2 min, followed by 25 cycles at 95 °C for 30 s, 50 °C for 30 s and 68 °C for 10 s, with a final extension at 68 °C for 5 min. The PCR products were separated on 1.6% agarose gels and visualized under UV light.

2.8. Freezing tolerance assays

Two-week-old *Arabidopsis* plants were removed from their agar plates and allowed to stand in water for 24 h. Then, the plants were pretreated with either 22 °C for 24 h (22 °C), 4 °C for 24 h (4 °C), 6% DMSO at 22 °C for 24 h (DMSO 22 °C), 40 mM BA at 22 °C for 1 h and then kept at 22 °C for 24 h (BA 22 °C), or 40 mM BA at 22 °C for 1 h and then shifted to 4 °C for 24 h (BA 4 °C). The pretreated plants were transferred to soil. Subsequently, the freezing tolerance of the plants was evaluated. One set of pretreated plants was incubated at −4 °C for 30 h in the dark (freezing treatment). Another set of pretreated plants was incubated at 22 °C (control condition). After the freezing treatments, the plants were grown at 22 °C in continuous light conditions.

3. Results

3.1. Membrane rigidification induces MPK4 activation during cold signaling

To investigate whether membrane rigidification affects cold signaling, *Arabidopsis* seedlings were treated with DMSO or BA as a membrane rigidifier and a membrane fluidizer, respectively. The kinase activity of immunoprecipitated MPK4 from cold- or DMSO-treated seedlings was measured using MBP as a substrate. MPK4 was activated by cold treatment in accordance with the previous reports (Fig. 1A and B) [8,10]. MPK4 activation was also detected in both 3% and 6% DMSO treatments without cold

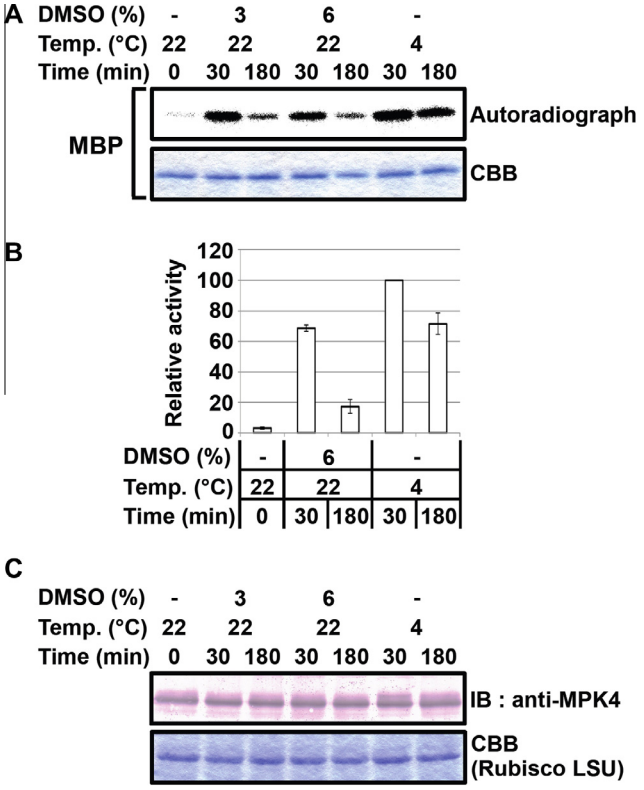

Fig. 1. DMSO treatment of *Arabidopsis* seedling induces the activation of MPK4. Total protein extracts were obtained from *Arabidopsis* seedlings subjected to 3% DMSO, 6% DMSO, or cold treatment for the indicated times. (A) Immunoprecipitated MPK4 was incubated with MBP in the kinase reaction mixture for 20 min, as described in the Section 2. The samples were separated using SDS–PAGE and subjected to autoradiography. Phosphorylation and Coomassie brilliant blue (CBB) staining of MBP are shown in the upper and lower panels, respectively. (B) The phosphorylation of MBP was quantified and the relative amounts of each phosphorylation were calculated when the MBP phosphorylation by the immunoprecipitated MPK4 from seedlings treated with cold for 30 min was 100. Vertical lines on each bar indicate ±SE ($n = 3$). (C) The extracts were separated by SDS–PAGE and subjected to immunoblot analysis with anti-MPK4 antibody (IB: anti-MPK4) and stained with CBB. The large subunit (LSU) of ribulose-1,5-bis-phosphate carboxylase/oxygenase (Rubisco) as the loading control are shown in CBB staining.

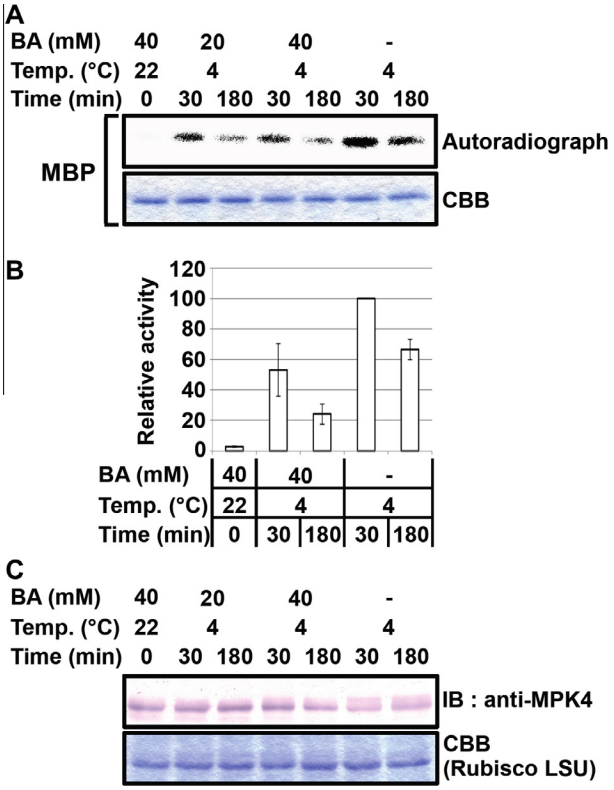

Fig. 2. BA inhibits cold-induced activation of MPK4. Total protein was extracted from *Arabidopsis* seedlings subjected to cold treatment with/without 20 mM or 40 mM BA pretreatment as shown. (A) The kinase activity of immunoprecipitated MPK4 was measured as described in Fig. 1A. (B) The phosphorylation of MBP was quantified and the relative amounts of each phosphorylation were calculated as described in Fig. 1B. (C) The extracts were subjected to immunoblot analysis with anti-MPK4 antibody and CBB staining as in Fig. 1B.

treatment (Fig. 1A and B). In both cold and DMSO treatments, the kinase activity of MPK4 after 30 min treatment was high compared to the 180 min treatment. Immunoblot analysis by using an anti-MPK4 antibody indicated that the amount of MPK4 protein did not change significantly during both cold and DMSO treatments (Fig. 1C). Seedlings were pretreated with the membrane fluidizer benzyl alcohol (BA) for 1 h before cold treatments. MPK4 was immunoprepicipated from cold-treated seedlings with/without BA pretreatment and subjected to a kinase assay. Cold-induced MPK4 activity decreased when seedlings were pretreated with 20 mM and 40 mM BA (Fig. 2A and B). Pretreatments with either concentration of BA were equally effective at reducing cold-induced MPK4 activity (Fig. 2A and B). BA pretreatment did not affect the amount of MPK4 protein (Fig 2C). These results suggest that membrane rigidification leads to MPK4 activation in cold signaling.

3.2. Membrane rigidification induces the phosphorylation of MKK2 and MEKK1 during cold signaling

We previously reported that immunoprecipitated MEKK1 phosphorylates MKK2 during cold treatment, and the degree of MKK2 phosphorylation by protein extracts from cold-treated seedlings reflects the activity of MEKK1 during cold treatment [10]. In addition, we reported that MEKK1 is also phosphorylated by protein extracts from cold-treated seedlings [10]. Thus, the kinase activity of the protein extracts from the DMSO- or BA-treated seedlings were measured using kinase negative forms of MKK1, MKK2 and MEKK1 as substrates. The protein extracts from 3% DMSO, 6% DMSO and cold-treated seedling phosphorylated both MKK2 and MEKK1 but not MKK1 (Fig 3). The cold-induced phosphorylation of MKK2 and MEKK1 was suppressed by 40 mM BA pretreatment (Fig 4). These results suggest that membrane rigidification acts upstream of the MEKK1-MKK2-MPK4 cascade during cold signaling.

3.3. Membrane rigidification induces the expression of COR15a

To investigate whether membrane rigidification is sufficient for the expression of cold-inducible genes, we analyzed the expression of *COR15a* in seedlings treated with DMSO or BA. It has been reported that the *COR15a* gene is expressed during cold signaling and it encodes a hydrophilic polypeptide [18]. The *COR15a* expressions were induced during cold treatments, and also induced in 6% DMSO treatments without cold treatment (Fig. 5). On the other hand, the expressions of *COR15a* during cold treatments were suppressed by 40 mM BA pretreatment (Fig. 5).

3.4. Membrane rigidification enhances freezing tolerance

We demonstrated that MPK4 activation, the phosphorylation of MKK2 and MEKK1, and the expression of *COR15a* were induced

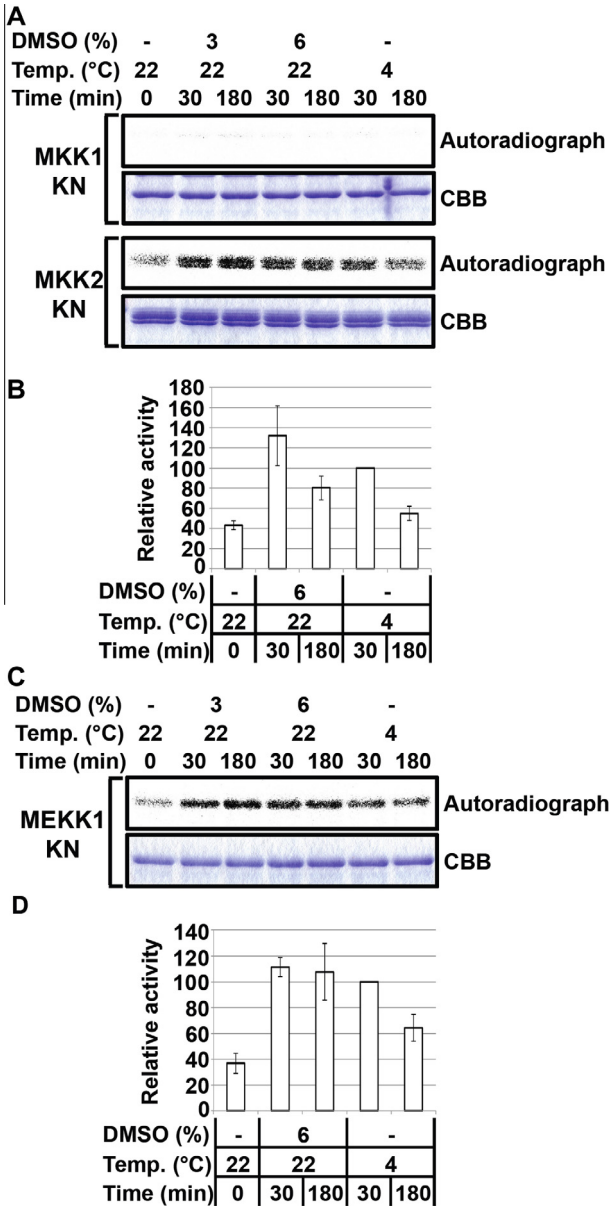

Fig. 3. Phosphorylation of MKKs and MEKK1 by an enzyme in the protein extracts from the seedlings subjected to DMSO treatment. The same protein extracts as in Fig. 1 were used. (A) Protein extracts (0.1 μg) were reacted with MKK1 KN or MKK2 KN as described in the Section 2. Phosphorylation and CBB staining of MKK1 KN or MKK2 KN are shown. (**B**) The phosphorylation of MKK2 was quantified and the relative amounts of phosphorylation were calculated when the MKK2 phosphorylation by the extracts from seedlings treated with cold for 30 min was 100. Vertical lines on each bar indicate ±SE ($n = 3$). (C) The same protein extracts were also reacted with MEKK1 KN as in Fig. 3A. Phosphorylation and CBB staining of MEKK1 KN are shown. (D) The phosphorylation of MEKK1 was quantified and the relative amounts of phosphorylation were calculated when the MEKK1 phosphorylation by the extracts from seedlings treated with cold for 30 min was 100. Vertical lines on each bar indicate ±SE ($n = 3$).

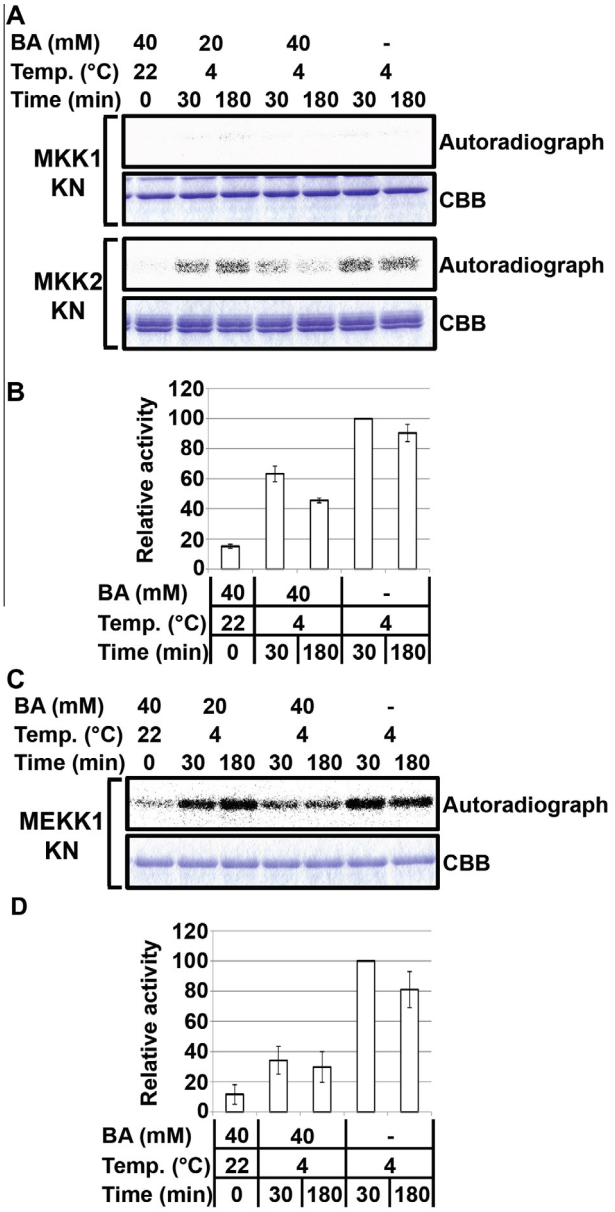

Fig. 4. Phosphorylation of MKKs and MEKK1 by an enzyme in the protein extracts from the seedlings subjected to cold treatments with BA pretreatment. The same protein extracts as in Fig. 2 were used. (A) The protein extracts were reacted with MKK1 KN or MKK2 KN as described in Fig. 3A. (**B**) The phosphorylation of MKK2 was quantified and the relative amounts of each phosphorylation were calculated as described in Fig. 3B. (C) The phosphorylation of MEKK1 KN by the same protein extracts was also monitored as in Fig. 3B. (**D**) The phosphorylation of MEKK1 was quantified and the relative amounts of each phosphorylation were calculated as described in Fig. 3D.

by DMSO treatment without cold treatment, whereas these cold-induced events were suppressed by BA pretreatment (Figs. 1–5). To investigate whether these reagents affect the acquisition of freezing tolerance, we evaluated the freezing tolerance of *Arabidopsis* plants pretreated by these reagents. One set of pretreated plants was incubated at −4 °C for 30 h in the dark (freezing treatment). Another set of pretreated plants was incubated at 22 °C (control condition). In the control condition, only plants pretreated with BA at 22 °C died (Fig. 6A). Plants pretreated with BA at 4 °C could grow under the control conditions, as well as plants pretreated with growth at 22 °C and 4 °C (Fig. 6A). DMSO-pretreated plants also could grow, but these were smaller than the 22 °C and 4 °C pretreated plants (Fig. 6A). When treated with freezing temperatures, 22 °C pretreated plants did not grow, whereas 4 °C pretreated plants showed freezing tolerance (Fig. 6B). This result is in accord with many previous reports [9,19,20] and indicates that *Arabidopsis* has a cold acclimation mechanism. Plants pretreated with DMSO exhibited freezing tolerance without 4 °C pretreatment (Fig. 6B). In contrast, plants pretreated with BA did not exhibit freezing tolerance regardless of pretreatment at 4 °C (Fig. 6B). These data suggest that the membrane rigidification plays

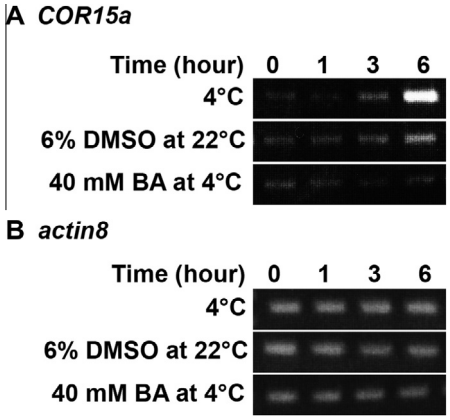

Fig. 5. Membrane rigidification induces the expression of *COR15a*. Two-week-old plants subjected to 4 °C and 6% DMSO at 22 °C treatments, respectively, for the indicated times, and also subjected to 40 mM BA at 22 °C for 1 h and then shifted to at 4 °C for the indicated times. Total RNA was extracted from these treated seedlings. (A) The expression levels of *COR15a* were analyzed using RT-PCR. (B) As a control, the expression levels of *actin8* were also indicated.

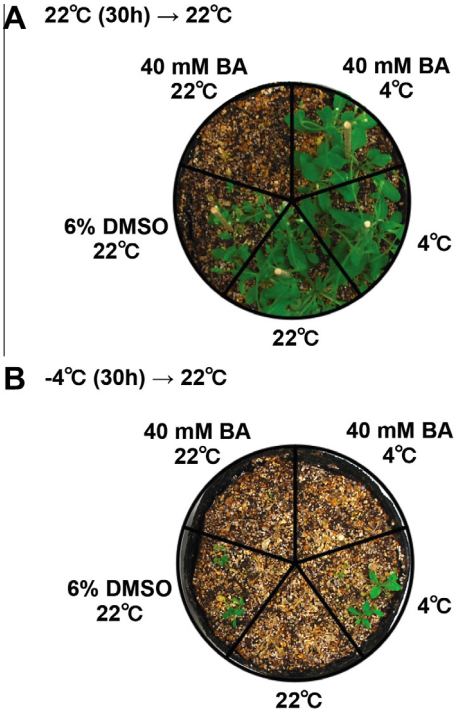

Fig. 6. Membrane rigidification contributes to enhance freezing tolerance. Two-week-old plants were pretreated with either 22 °C for 24 h (22 °C), 4 °C for 24 h (4 °C), 6% DMSO at 22 °C for 24 h (6% DMSO 22 °C), 40 mM BA at 22 °C for 1 h and then kept to 22 °C for 24 h (40 mM BA 22 °C), or 40 mM BA at 22 °C for 1 h and then shifted to at 4 °C for 24 h (40 mM BA 4 °C). The pretreated plants were transferred to soil, then treated at 22 °C for 30 h in normal light condition (A) or −4 °C for 30 h in the dark (B). Pictures were taken 12 days at 22 °C in normal light conditions after each treatment.

a key role in the cold acclimation required to enhance the freezing tolerance of *Arabidopsis*.

4. Discussion

In this study, we showed that DMSO treatment of *Arabidopsis* seedlings caused the activation of MPK4 without cold treatment (Fig. 1), while MPK4 activation under cold conditions was suppressed by BA pretreatment (Fig. 2). MKK2 and MEKK1 were phosphorylated by extracts from DMSO-treated seedlings, whereas the phosphorylation of MKK2 and MEKK1 by the extracts from cold-treated seedlings was suppressed with the BA pretreatment (Figs. 3 and 4). These results suggest that the cold-induced membrane ridigification takes place upstream of the MEKK1-MKK2-MPK4 pathway. It has been reported that MEKK1 is phosphorylated via Ca^{2+} signaling in the cold response and that CRLK1 is the one of candidates located upstream of MEKK1 [10]. It has also been reported that cold treatment induces a Ca^{2+} influx via membrane rigidification in alfalfa cells [21]. Taken together, the following signaling model is suggested. Cold conditions induce membrane rigidification, which prompts a Ca^{2+} influx. The rise in the concentration of intracellular Ca^{2+} leads to the activation of a signaling pathway, the Ca^{2+}/CaM-CRLK1–MEKK1-MKK2-MPK4 pathway. It was reported that both cold treatments and membrane rigidification activates the HOG1 MAPK in *Saccharomyces cerevisiae* [22], suggesting that the similar pathways may act during cold signaling in both yeast and plant.

The DMSO-treated *Arabidopsis* plants displayed freezing tolerance without cold acclimation, whereas BA pretreatment during cold acclimation preventing plants from acquiring freezing tolerance (Fig. 6). In addition, previous research has shown that plants overexpressing constitutively activated MKK2 show freezing tolerance without cold acclimation, whereas *mkk2* null plants are hypersensitive to freezing temperatures [9]. These results indicated that the MEKK1-MKK2-MPK4 cascade functions in the acquisition of freezing tolerance through membrane rigidification in *Arabidopsis*. Furthermore, plants overexpressing constitutively activated MKK2 show up-regulation of *CBF/DREB1* genes, which are transcription factors that play an important role in acquiring freezing tolerance [9]. Moreover, we indicated that the cold-induced gene, *COR15a*, is up-regulated by membrane rigidification (Fig. 5). The *COR15a* is one of the downstream targets of CBF/DREB1 [2]. These observations suggest that the CBF pathway is located downstream of this MAPK cascade in the cold acclimation response via membrane rigidification.

Although *Arabidopsis* plants treated with DMSO at 22 °C could grow in both the control conditions and the freezing-treatment conditions, these plants exhibited a smaller phenotype in size than other non-DMSO treated plants (Fig. 6). Intracellular DMSO may affect the growth of these plants. Plants treated with BA at 22 °C could not grow even in the control conditions, whereas plants treated with BA at 4 °C could grow in the control conditions (Fig. 6). It has been reported that the membrane fluidity that is increased by BA treatment at normal growth temperatures mimics heat treatment in alfalfa cells [15]. In this study, treatment of *Arabidopsis* plants with BA at 22 °C may mimic heat treatment, with the result that these plants could not survive. However, plants treated with BA at 4 °C could grow in the control conditions. Plants treated with BA at 4 °C may maintained their membrane fluidity at the same level as plants grown at 22 °C in the absence of BA.

In this report, we suggest that membrane ridigifiction occurs upstream of the MEKK1-MKK2-MPK4 cascade in the cold response and is necessary to acquire freezing tolerance. Further investigation to identify molecules that are involved in sensing the downshift in the membrane fluidity may elucidate the mechanism of cold acclimation in higher plants.

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