Calcium/calmodulin-regulated receptor-like kinase CRLK1 interacts with MEKK1 in plants

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Recently we reported that CRLK1, a novel calcium/calmodulin-regulated receptor-like kinase plays an important role in regulating plant cold tolerance. Calcium/calmodulin binds to CRLK1 and upregulates its activity. Gene knockout and complementation studies revealed that CRLK1 is a positive regulator of plant response to chilling and freezing temperatures. Here we show that MEKK1, a member of MAP kinase kinase kinase family, interacts with CRLK1 both in vitro and in planta. The cold triggered MAP kinase activation in wild-type plants was abolished in crlk1 knockout mutants. Similarly, the cold induced expression levels of genes involved in MAP kinase signaling are also altered in crlk1 mutants. These results suggest that calcium/calmodulin-regulated CRLK1 modulates cold acclimation through MAP kinase cascade in plants.

Calcium, a universal second messenger in eukaryotic cells, mediates changes in external and internal signals leading to the physiological responses.1-4 Calcium/calmodulin (Ca2+/CaM)-dependent protein kinases (CaMKs) are very important players in calcium/calmodulin mediated signaling in mammalian cells.5 In plants, Ca2+/CaM-dependent protein phosphorylation was observed more than 25 years ago.6 Several calmodulin-regulated protein kinases have been identified and characterized.7,8 For example, plants have a unique chimeric Ca2+/CaM-dependent protein kinase (CCaMK), which exhibits Ca2+-dependent autophosphorylation and Ca2+/CaM-dependent substrate phosphorylation.9 CCaMK is required for bacterial and fungal symbioses in plants.10-12 Recently, we characterized a novel plant-specific calcium/CaM-regulated receptor-like kinase, CRLK1.13 Ca2+/CaM binds to CRLK1 and stimulates its kinase activity. Functional studies with CRLK1 indicate that CRLK1 acts as a positive regulator in plant response to chilling and freezing temperatures. To further define the CRLK1-mediated signal pathway, we isolated CRLK1 interacting proteins by co-immunoprecipitation using an anti-CRLK1 antibody. Since cold increases the amount of CRLK1 protein, wild-type plants (WT) were treated at 4°C for 1 hr before co-immunoprecipitation. The resulting CRLK1 immunocomplex was separated by SDS-PAGE. We observed several bands of different sizes only in the wild-type but not in the crlk1 knockout mutant plants (Fig. 1A).

Furthermore, the intensity of these bands increased upon cold treatment, suggesting that they are the putative partners or associated proteins of the CRLK1 immunocomplex.

To determine the identities of these proteins, mass spectrometric analysis was performed with the total immunocomplex.14 In addition to CRLK1, there were 12 other proteins which matched the Arabidopsis database. Several of them appeared in the pull-down complex from WT, but not from crlk1 mutants. These putative interacting proteins included MEKK1, another unknown protein...
CRLK1 interacts with MEKK1 in vitro and in planta

To confirm the direct interaction between CRLK1 and MEKK1, a well-characterized component in cold signaling, we performed GST pull-down assay (Fig. 1C). The recombinant CRLK1 M29–440 was precipitated by GST:MEKK1, but not by GST alone. However, the intensity of the band was very low, suggesting weak interaction between them. Since CRLK1 is a calcium/CaM-regulated kinase, we investigated the effects of calcium and/or CaM on the interaction between CRLK1 and MEKK1. In the presence of calcium and CaM in the reaction mixture, the interaction between CRLK1 and MEKK1 was dramatically increased as reflected by the intensity of the band (Fig. 1C). These results indicate that the binding of calcium/CaM to CRLK1 increases its affinity to MEKK1.

To address if CRLK1 and MEKK1 associate in vivo and to determine subcellular location of this association, we used Bimolecular Fluorescence Complementation (BiFC) in Arabidopsis protoplasts.22 BiFC vectors carrying CRLK and MEKK1 were co-transfected into protoplasts and observed for the reconstitution of YFP fluorescence. Confocal images showed that CRLK1 and MEKK1 associate both on cell membrane and in endosomes. The middle and last rows are controls. Bar = 10 µm.

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**Figure 1.** CRLK1 interacts with MEKK1. (A) One-dimension SDS-PAGE of anti-CRLK1 immunocomplexes from 3-week-old WT or crkl1 plants with or without cold treatment. One mg of total protein was used for immunoprecipitation. (B) A list of putative CRL1-interacting proteins determined by MALDI-TOF-MS analysis. (C) CRLK1 interacts with MEKK1 as shown by GST pull-down assay. (D) BiFC analysis show that CRLK associates with MEKK1 in vivo. Upper row shows that CRLK and MEKK1 associate both on cell membrane and in endosomes. The middle and last rows are controls. Bar = 10 µm.
Loss of CRLK1 Altered MAP Kinase Activity and Expression Levels of Genes Involved MAPK Pathway

To further study the relationship between CRLK1 and MAPK signaling, we compared the MAPK activity between WT and crlk1 plants in response to cold treatment using in gel phosphorylation assay (Fig. 2A). In WT plants, cold stimulated MAP kinase activity. However, this stimulation was diminished in crlk1 mutants. These results suggest that CRLK1 plays a role in regulating the MAPK cascade during cold signaling.

It has been shown that cold treatment increases the expression of MEKK1. To investigate whether CRLK1 affects MEKK1 expression, we compared the RNA level of MEKK1 between WT and crlk1 mutants using semi-quantitative RT-PCR. MEKK1 levels in both WT and crlk1 plants were similar after cold treatment, suggesting that CRLK1 does not regulate MEKK1 at the transcriptional level (data not shown). We further studied the expression of the marker genes such as RAV1, RAV2 and STZ affected by MAPK cascade. Their expression levels were lower in crlk1 plants as compared to WT after cold treatment (Fig. 2B). These results are consistent with our earlier observation that CBF and COR genes expression were reduced or delayed in crlk1 knockout plants as compared to wild-type plants during cold treatment.

It is documented that cold responsive CBF and COR genes expression are regulated by MAPK pathway.

Accumulating evidence indicates that protein phosphorylation is involved in the pathway connecting the cold-triggered calcium changes and cold acclimation. However, the protein kinase(s) responsible for inducing cold-regulated genes and activating freezing tolerance is elusive. There are interesting candidates such as an alfalfa MPK, p44mkk4, that is activated within 10 min after being exposed to low temperature. Expressing a heterologous tobacco MAPKK (Nicotiana PK1) enhances freezing tolerance in transgenic maize plants that are normally frost sensitive. In Arabidopsis, MEKK1, MKK2, MPK4 and MPK6 have been shown to be involved in cold signal transduction.

Our results suggest that calcium/CaM/CRLK1 interacts with MEKK1 and regulates the MAPK cascade during cold stress. Figure 2C is a working model showing the components of CRLK1-mediated cold stress signal transduction pathway linking calcium/calmodulin and MAPK cascade. Further studies on the hierarchy of calcium/CaM/CRLK1 and MAPK will shed light on our understanding of the mechanisms of cold signal transduction pathway(s) in plants.

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References


