# Characterization of Calreticulin as a Phosphoprotein Interacting with Cold-Induced Protein Kinase in Rice

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**Calreticulin is an abundant endo/sarcoplasmic reticulum $Ca^{2+}$-binding protein. To investigate whether calreticulin (CRO1) is involved in the cold-stress response in rice, a transgenic plant was constructed. The transcriptional level was decreased within 30 min and recovered within 2 h of a cold treatment. The calreticulin protein was shifted from a soluble fraction to an insoluble fraction by cold stress. Endogenous abscisic acid (ABA) is an important factor in cold response, and the synthesis of ABA was strongly induced in CRO1-sense transgenic rice, the same as in cold-sensitive rice. The phosphorylation of calreticulin increased after cold treatment. Overexpression of calreticulin enhanced the activities of 47 kDa $Ca^{2+}$-dependent protein kinase (CDPK) that had been induced by cold treatment. The 47-kDa CDPK activity increases more in the cold sensitive variety IR36 and the sense transgenic rice than it does in other varieties. The synthesis of ABA, phosphorylation of calreticulin and 47-kDa CDPK activity induced in sense transgenic rice were the same as in cold-sensitive rice and the phosphorylation of antisense transgenic rice was similar to that of cold-tolerant rice. These results suggest that the calreticulin is involved in the signaling pathway leading to response to cold stress.**

**Key words** abscisic acid; calreticulin; $Ca^{2+}$-dependent protein kinase; cold stress; protein phosphorylation; rice

Cold stress is associated with rapid and reversible changes in the phosphorylation status of specific pre-existing protein and $Ca^{2+}$ plays a key role in this process.[1] Previous studies indicated the effects of cold stress on protein phosphorylation in rice varieties differing in cold susceptibility.[2,3] It appears that the $Ca^{2+}$ and protein phosphorylation-mediated pathway participate in low-temperature signal transduction. Calreticulin is an abundant endo/sarcoplasmic reticulum protein located primarily within the lumen of endoplasmic reticulum (ER), although it is also found at other cellular locations such as nucleus and plasma membrane.[4] Calreticulin is involved in regulating intracellular $Ca^{2+}$ signaling and acts as a chaperone assisting in protein processing.[5] There are evidences for the participation of calreticulin in $Ca^{2+}$- and phospholipid-dependent protein kinase (PKC) and inositol signaling transduction pathway.

$Ca^{2+}$-dependent protein kinases (CDPKs) play an important role in plant stress signal transduction. Our previous studies indicated that a 47-kDa CDPK was induced by cold treatment.[6] It was found that calreticulin could interact with PKC *in vitro* and *in vivo*, suggesting that in plants calreticulin have a relationship with CDPK. In plants, CDPK is stimulated by an increase in $Ca^{2+}$ that results from various stimuli. Cold stress has been shown to induce $Ca^{2+}$ in cytosol,[7,8] and $Ca^{2+}$ could play an essential role in the cold acclimation.[9]

Denecke *et al.*[10] reported that the calreticulin in tobacco plants showed stress- and hormone-specific regulation. The plant hormone abscisic acid (ABA) plays an important role in mediating responses to environmental stress, including cold. ABA has been shown to accumulate in plant tissue in response to low temperature,[11] and many genes are responsive to exogenous application of ABA.[12] Plants must adjust their physiology to changes in environmental conditions in order to prevent damage and ensure survival. Temperature is one of the most variable environmental factors, to which plants are exposed. Changes in temperature affect many metabolic processes in the plant cell, and that may also modify its structural components. In rice plant, the growth rate and metabolism are markedly inhibited at the chilling temperature, but the mechanism for the effects of cold stress on growth and the accompanying metabolic changes remain unclear. During responses to cold stress, a variety of changes including alterations in lipid, protein and carbohydrate compositions are produced in the cells.[13] Cold can also induce acclimation-related proteins in many plants and it is generally recognized that the protein synthesis patterns and mRNA levels change when plants are exposed to low temperatures.[14]

A-56 kDa phosphoprotein purified from rice-cultured cells[15] was found to be involved in regeneration and identified as a rice calreticulin.[16] Calreticulin may play an important role as a signaling molecule during cell growth. For the first time, the data in the present study demonstrated that rice calreticulin phosphorylation states were affected by cold stress. Furthermore, calreticulin affected endogenous ABA concentrations and a cold-induced 47-kDa CDPK activity was discussed.

## MATERIALS AND METHODS

**Plant Materials** Rice (*Oryza sativa* L. cv. Nipponbare, IR36 and Kitaibuki) seedlings were grown under white fluorescent light (12 h light period/d) at 25 °C and 70% relative humidity in a growth cabinet for 2 weeks. For cold treatment, the leaf segments were incubated on 10 ml distilled water in 60×15 mm Petri dishes in a growth chamber at 5 °C under a 12 h photoperiod prior to sampling.

**Transformation of Rice Mediated by Agrobacterium** *Agrobacterium tumefaciens* strain EHA101 (a gift from Dr. E. Hood) has been described previously.[17] Plasmids were introduced into this strain by electroporation. *A. tumefaciens* was grown on AB medium at 28 °C.[18] The rice calreticulin gene (CRO1) was connected to 35S promoter in pIG121Hm (a gift from Dr. K. Nakamura), a binary vector that contains a kanamycin resistance gene (*npt*) and a hygromycin resis-

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tance gene (*hpt*), and the intron-gus in the T-DNA region. The transformation was done as previously reported.[19] The regenerated rice was eventually transferred to soil in pots and grown to maturity in a greenhouse.

**Quantitative RT-PCR** RT-PCR was performed using total RNA isolated from cold-treated (5 °C) rice leaf with a StartaScript™ RT-PCR Kit (Stratagene, La Jolla, CA, U.S.A.). The following primers were used to amplify and quantify the relative levels of the CRO1 mRNA: CAS-1 (sense, 5′-GAG GTC TTC TTC CAG GAG AAG TTC GAA GAC G-3′) and CAA-1 (antisense, 5′-TTG GGA ATA TCA TCA TAG CCC TC-3′). Actin 1 cDNA was co-amplified as the internal standard. Primers for Actin 1 were ACT-A (5′-ACT GTC CCC ATC TAT GAA GGA-3′) and ACT-B (5′-CTG CTG GAA TGT GCT GAG AGA-3′) (amplifying a fragment of 579 bp). Each RT-PCR reaction started with a total volume of 50 $\mu$l in the buffer recommended by the manufacture of the StartaScript™, contained 1 $\mu$g RNA, 10 U reverse transcriptase, 40 U RNase inhibitor, 25 pmol poly (dT) primer and 200 $\mu$M dNTPs. Incubation was carried out at 37°C for 1 h followed by a PCR cycling regime of 25 cycles at 95°C for 1 min, 54°C for 2 min, and 72°C for 3 min on a GeneAmp PCR system (Perkin Elmer, Foster City, CA, U.S.A.). The reaction contained primers (1 $\mu$M each), dNTPs (0.2 mM each) and 2.5 units of AmpliTaq in a 50 $\mu$l reaction volume. After 25 cycles, the amplified products were separated by agarose-gel electrophoresis and stained with ethidium bromide.

**Protein Extraction and Immunoblot Analysis** Total protein was extracted from 500 mg leaf or leaf sheath by grinding in 1 ml sodium dodecyl sulfate (SDS) sample buffer. The following procedures were carried out at 4 °C. For preparation of cytosolic and membrane fractions, the tissues were homogenized with 1 ml homogenization buffer containing 20 mM Tris–HCl (pH 7.5), 0.25 M sucrose, 10 mM EGTA, 1 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride.[20] The homogenates were centrifuged at 800×$g$ for 5 min and the supernatants were centrifuged at 100000×$g$ for 15 min at 4 °C. The cytosolic fraction was obtained by collection of the supernatant. The pellet was resuspended in 100 $\mu$l homogenization buffer and washed by centrifugation at 100000×$g$ for 15 min. The pellet was resuspended in 60 $\mu$l membrane solubilizing buffer containing 1% Triton X-100, 20 mM Tris–HCl (pH 7.5), 1 mM EDTA and 50 mM 2-mercaptoethanol utilized for 30 min on ice. The membrane fraction was obtained from the supernatant after centrifugation at 100000×$g$ for 7 min at 4 °C. The activity of ATPase as a marker enzyme was determined both in soluble and membrane fractions. The protein concentration was measured using a protein assay kit (Bio-Rad, Richmond, CA, U.S.A.). Immunoblotting analysis was performed as described previously.[15]

**Quantitative Determination of Endogenous ABA** The endogenous ABA concentration in leaf was detected using the Phytodetek enzyme immunoassay kit (Agdia, Elkhart, IN, U.S.A.) according to the protocol provided. The leaf segments were treated at 5 °C for 24 h, and at 25 °C for 24 h as control.

***In Vitro* Protein Phosphorylation** Five microliters protein crude extracts were incubated for 10 min at 30 °C in a total volume of 20 $\mu$l reaction mixture containing 20 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 0.2 mM $CaCl_2$ and 39 $\mu$M [$\gamma$-$^{32}$P]ATP (3000 Ci/mmol, Amersham Pharmacia, Buckinghamshire, U.K.). The basal level of phosphorylation was measured in the presence of 1 mM EGTA instead of $CaCl_2$. The phosphorylated proteins were separated by 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE). The gels were stained with Coomassie Brilliant blue (CBB), destained, dried and exposed to an X-ray film (Kodak, Rochester, NY, U.S.A.) at −80 °C for 5 to 7 d.[20]

**In-Gel Kinase Assay** Protein extracts were separated by 17% SDS polyacrylamide gels embedded with 2 mg/ml histone III-S (Sigma, St. Louis, MO, U.S.A.) as the substrate for the protein kinase. After electrophoresis, the SDS was removed by washing the gel with a buffer containing 50 mM Tris–HCl (pH 8.0) and 20% 2-propanol for 1 h, and then washed with 50 mM Tris–HCl (pH 8.0) solution containing 5 mM 2-mercaptoethanol for 1 h. The separated proteins were denatured for 1 h in 6 M guanidine–HCl, and then allowed to renature in 0.04% (w/v) Tween-40 at 4 °C for 16 h. The reaction was initiated by the addition of 5 $\mu$M [$\gamma$-$^{32}$P]ATP (3000 Ci/mmol, Amersham Pharmacia) in 40 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 2 mM DTT and 0.2 mM $CaCl_2$ at room temperature for 30 min. The reaction was stopped by extensive gel washing with 5% (w/v) trichloroacetic acid containing 1% (w/v) potassium PPi until background radioactivity decreased. The gels were stained with CBB, destained, dried and exposed to X-ray film (Kodak) at −80 °C for 3 d.

## RESULTS AND DISCUSSION

There is already an evidence that the concentration of internal $Ca^{2+}$ and ATP affects the association of calreticulin with some proteins such as BiP, PDI, calnexin and other peptides.[22] The binding site in calreticulin was localized to the proline-rich P-domain containing two sequence motifs and a high affinity $Ca^{2+}$ binding site,[16] which shows that calreticulin might be involved in the regulation of cellular or intercellular $Ca^{2+}$ signaling. A high capacity $Ca^{2+}$-binding protein calreticulin was detected in the lumen of the endoplasmic reticulin of a wide variety of cells.[4] Recent studies indicated that calreticulin is not only a $Ca^{2+}$-binding protein, but can also work as a multifunctional protein. For clarifying the role of calreticulin in cold response, the transcription level was determined using quantitative RT-PCR. The Actin 1 gene amplified under the same conditions was used in a constitutive expression control. Total RNA samples were prepared from leaf and leaf sheath that had been treated at 5 °C in a 6 h time course experiment for determining the transcriptional level of calreticulin after a cold treatment. The amount of calreticulin mRNA decreased within 30 min and recovered within 2 h of the cold treatment in both leaf and leaf sheath tissues (Fig. 1A). The calreticulin is affected by cold at a very early stage and the sudden decrease of calreticulin mRNA, suggesting that the normal transcription factors of calreticulin are very sensitive to low temperature. The mRNA level seems to be recovered within 2 h (Fig. 1A); however, it is still less than the normal level when compared to the 25 °C control. Cold stress can affect the expression of many genes, and the proteins encoded by these genes are thought to function in protecting cells from the stress.[23] In the present study, the transcription of calreticulin appears to

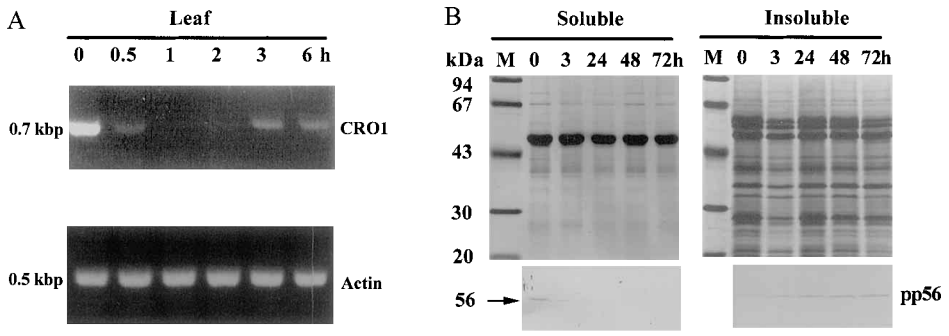


Fig. 1. Effect of Cold Stress on the Transcription Levels and the Distribution of Rice Calreticulin

(A) The total RNA was prepared from leaf tissue treated at 5 °C with the time course (0, 0.5, 1, 2, 3 and 6 h). The expression levels of rice calreticulin (CRO1) were analyzed by quantitative RT-PCR as described in Materials and Methods. The products were separated by agarose-gel electrophoresis and stained with ethidium bromide. Actin1 gene product was amplified under the same condition as a constitutive expression control. The sizes of CRO1 and Act1 fragments are indicated as 0.7 kbp and 0.5 kbp. B. Immunoblot analysis of calreticulin in leaf after cold treatment. The subcellular fractions were prepared from rice leaf tissue treated at 5 °C with the time course (0, 3, 24, 48, 72 h). ATPase activity was determined to be 12.9 mg/mg in soluble fraction and 129.8 mg/mg in the membrane fraction (phosphoric acid/protein). An equal amount of protein (20 $\mu$g per each lane) was separated by SDS-PAGE. Immunoblot analysis was performed with anti-pp56 antibody as described in Materials and Methods. CBB staining patterns were shown as protein concentration control. The position of calreticulin is indicated as pp56 (56 kDa).

respond to a cold treatment that inhibits the transcription in very short time, but does not stop it. This result suggests that in rice cells the calreticulin gene has some cold-stress inducible-transcription factors that involved in the maintenance of the calreticulin mRNA under cold treatment.

The protein levels of rice calreticulin were measured in leaf after cold treatment were measured using immunoblotting analysis. The leaf was treated at 5 °C in a 72 h time course, and the proteins were separated into soluble and insoluble fractions, and the amount of calreticulin was determined with immunoblot analysis. ATPase activity was determined to be 12.9 mg/mg in soluble fraction and 129.8 mg/mg in the membrane (phosphoric acid/protein). After cold treatment, the calreticulin decreased in soluble fractions and increased in insoluble fractions (Fig. 1B). The result implied a traslocation of calreticulin in the leaf and leaf sheath. These results suggest that the calreticulin response to the cold stress might be mediated by a translocation or by the presence of signaling mechanisms between the membrane and cytosol. In mammalian cells, calreticulin is transported to the plasma membrane and tightly binds to the surface molecule. One of the properties of calreticulin is that it functions as a molecular chaperone. This is further supported by studies demonstrating that the function of calreticulin is the same as that of calnexin, which shows a homology to calreticulin and contains the same repetitive motifs,[13] in promoting the efficient folding of proteins.[29] Cold treatment may selectively release the peripheral subunits from some membrane protein complexes (*e.g.* ATPase complex) and partially degrade those complexes. Calreticulin was also found in association with BiP (rumenal binding protein) in plants.[24] Both BiP and calnexin were found in association with $H^+$- ATPase.[25] It is reasonable that calreticulin might be required in this process. Further studies that focus on these interactions will give a better understanding of calreticulin in response to cold stress.

In order to know precisely the function of calreticulin in rice tissues after cold stress, the full-length cDNA for CRO1[16] was introduced into rice cells (cv. Nipponbare) in the sense and antisense orientation under the control of the cauliflower mosaic virus (CaMV) 35S promoter in the pIG121-Hm vector by means of *Agrobacterium*-mediated transformation.[19] Then, 20 independent lines of transgenic

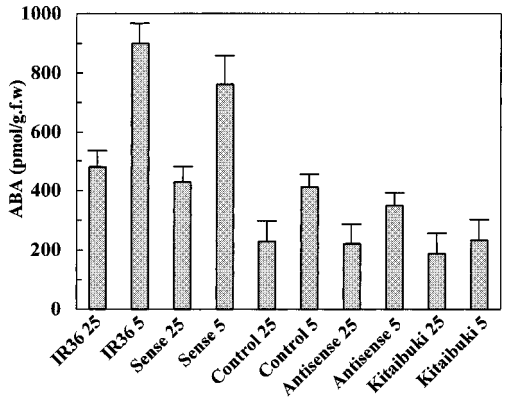


Fig. 2. Changes of Endogenous ABA Concentration in Leaf of Different Rice Varieties after Cold Stress

Rice leaves were treated at 25 °C and 5 °C for 24 h, and the endogenous ABA concentration was detected using the Phytodetek enzyme immunoassay kit.

plants were generated and confirmed with immunoblotting (data not shown). One line of over-expressing CRO1, Sense-1 and one-line antisense, Antisense-1, were chosen for further experiments. Calreticulin was reported as a negative regulator in mammalian cells.[21] In our previous research, calreticulin negatively affected the rice callus regeneration and growth.[16] In the present study, the over-expression of calreticulin inhibited the callus regeneration and seedling growth speed (data not shown). This transgenic rice of CRO1 was used for clarification of the role of calreticulin in rice plant.

It has been suggested that the phytohormone ABA has an important role in the enhancement of the freezing tolerance of cold-acclimated plants. ABA levels increase in certain plants in response to cold.[26—28] While there are several studies suggesting that ABA has an important role in cold acclimation, the evidence remains circumstantial. Calreticulin is proposed to have several functions including a role in $Ca^{2+}$-binding and storage, and $Ca^{2+}$-signaling. These properties suggest that calreticulin may mediate the free $Ca^{2+}$ in the ABA pathway in response to cold stress. The ABA level in rice leaf was determined after cold treatment. After treatment at 5 °C for 24 h, the ABA concentration in rice leaf increased in some varieties and calreticulin in transgenic rice (Fig. 2). The cold-sensitive variety IR36 and CRO1-sense transgenic

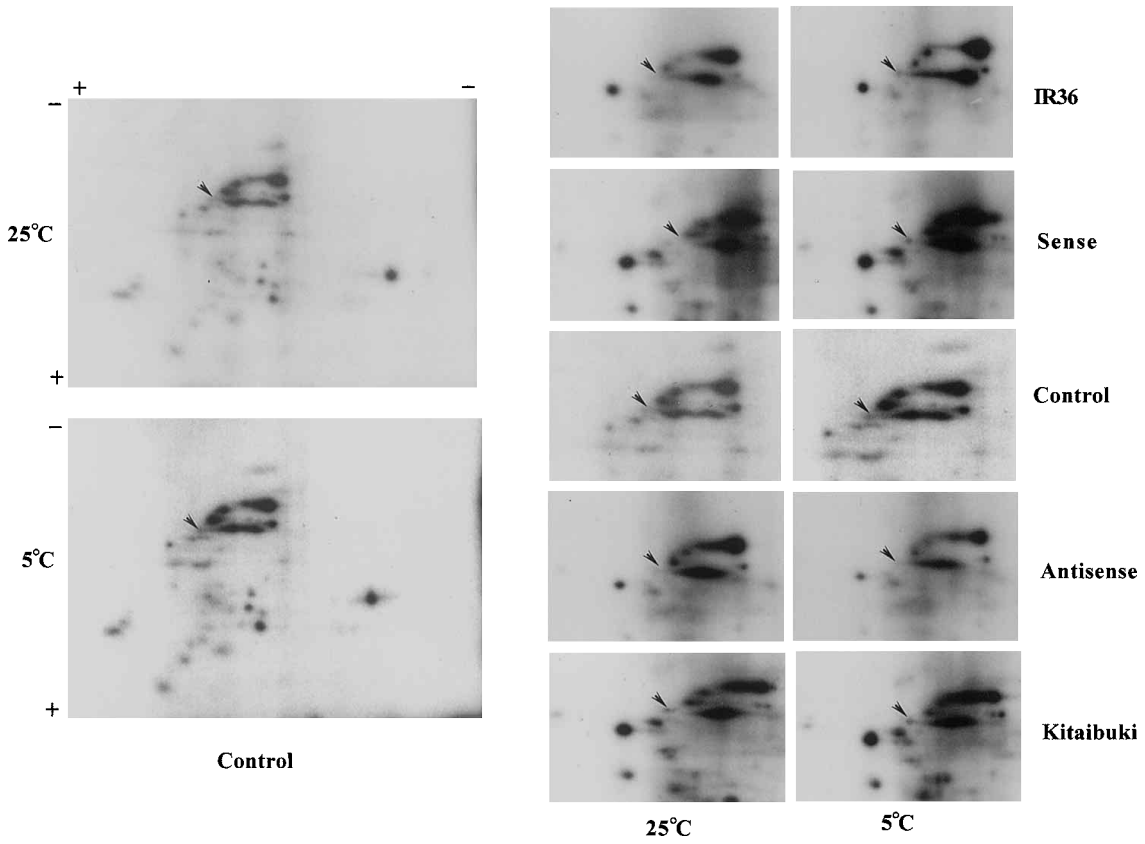


Fig. 3. *In Vitro* Protein Phosphorylation of Calreticulin in Rice Leaf after Cold Treatment

Proteins were extracted from leaf treated for 1 h at 5 °C and 25 °C as control. Protein extracts were phosphorylated with [$\gamma$-$^{32}$P]ATP containing 0.2 mM $CaCl_2$. The phosphorylated proteins were separated by 2D-PAGE, and exposed to an X-ray film. The arrow shows the position of rice calreticulin. The position of rice calreticulin was detected by immunoblot analysis using anti-pp56 antibody.

rice showed more endogenous ABA than cold-insensitive variety Kitaibuki and CRO-1 antisense transgenic rice. Furthermore, the cold induces more ABA in IR36 and sense transgenic seedling leaves (Fig. 2). These results indicate that the calreticulin participated in the cold stress response in a manner relative to the ABA.

Calreticulin has been found as phosphoproteins from rice-cultured suspension cells,[15] and it has been identified and characterized as a functional protein involved in the regeneration of rice-cultured suspension cells.[16] In other reports, calreticulin has been identified in a search for oligogalacturonide-modulated phosphoproteins.[29] Spinach calreticulin was phosphorylated specifically by protein kinase CK2 *in vitro*.[30] To provide additional evidence for possible role of rice calreticulin in cold treatment, the phosphorylation status of calreticulin under cold treatment conditions was analyzed using an *in vitro* phosphorylation assay. The phosphorylation of calreticulin increased after the treatment of leaf tissue at 5 °C for 60 min. Adding 1 mM EGTA to the reaction buffer inhibited the phosphorylation of calreticulin, and the additional 0.2 mM $CaCl_2$ increased the phosphorylation of calreticulin (data not shown). These results indicate that the cold treatment affected the protein phosphorylation status of calreticulin as well as quantity of protein. Furthermore, the phosphorylation of transgenic rice were analyzed under the same conditions. The CRO1-sense transgenic rice has an unusual ability to phosphorylate calreticulin, particularly when compared to IR36 and Kitaibuki (Fig. 3). An essential aspect of future research will be to find the putative kinase responsible for the phosphorylation of calreticulin in subcellular fractions in response to cold stress.

For understanding the role of calreticulin in response to cold stress, transgenic rice with calreticulin cDNA was used to perform an in-gel kinase assay. The in-gel kinase assay has proven to be useful in the detection of protein kinases as well as in the study of their roles in several physiological processes of plants.[30] In this experiment, soluble and insoluble fractions, prepared from normal or transgenic rice leaves treated at 5 or 25 °C for 1 h, were separated by SDS-polyacrylamide gel containing histone III-S as a substrate. [$\gamma$-$^{32}$P] ATP was added to allow for the detection for phosphorylation. The proteins at 17-, 47- and 55-kDa in insoluble fractions, respectively, had kinase activities either in control, sense and antisense rice leaves (Fig. 4A). The 47- and 55-kDa protein kinase showed the $Ca^{2+}$-dependent kinase activities because they were completely inhibited by the 1 mM EGTA substitute for $Ca^{2+}$ (Fig. 4A lower). In sense transgenic rice the activities of 47-kDa protein kinase are stronger than in the control (about 2 fold) either treated at 5 or 25 °C, and these 2 protein kinase activities were clearly induced by cold treatment. The activities in both 47- and 55-kDa protein kinases were about two times stronger than in the control. Furthermore, the over-expression of calreticulin specifically enhances the activity of 47-kDa CDPK (Fig. 4B). The cold sensitive varieties IR36 and CRO1 sense transgenic rice have a larger increase of 47 kDa CDPK activities than antisense

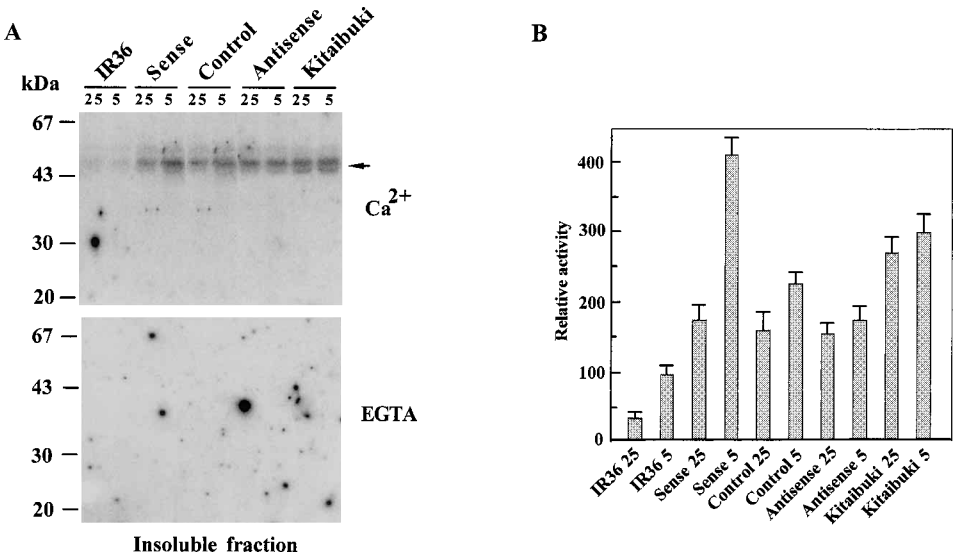


Fig. 4. Over-Expression of Calreticulin Stimulated Cold Induced 47 kDa CDPK Activity

(A) In-gel kinase assay using rice leaves after cold treatment. The leaves were treated for 1 h at 5 or 25 °C. The IR36 and Kitaibuki were treated at the same condition as transgenic rice. The insoluble fractions from leaves were separated by SDS-PAGE containing 2 mg/ml histone III-S. In-gel kinase assay was performed in the presence of 0.2 mM $CaCl_2$ (upper) or 1 mM EGTA (lower). Arrowhead shows the position of the CDPK (47 kDa). (B) Radioactivities in each lane in A were taken as the signals of 47 and 55 kDa CDPKs. A bar with an error bar indicates the average value derived from three independent series of samples, and a gel profile from one of them is shown in A.

transgenic rice and the cold-tolerant variety Kitaibuki (Fig. 4B). These results suggested that calreticulin might regulate CDPK activity. The calreticulin promotion of the $Ca^{2+}$-dependent protein kinase activity might be a trigger of response to cold stress in rice. In over-expression of calreticulin in the mouse L fibroblast cell line, the total cellular $Ca^{2+}$ content was found to have about 2-fold increase, and the cytosolic free $Ca^{2+}$ elevations were enhanced when either ATP or a combination of ionomycin and thapsigargin was used as a stimulus.[31] $Ca^{2+}$ binding to calreticulin may regulate the free $Ca^{2+}$ concentration and the level of free calreticulin. The present results suggested that the calreticulin does respond to cold stress. Furthermore, the response may be through the $Ca^{2+}$ signal transduction pathway, and the phosphorylation and dephosphorylation of calreticulin might be the trigger of those processes.

The results show that the rice calreticulin is involved in the response to cold stress. Although the calreticulin protein shows no significant changes in amount, the distribution of calreticulin is changed by cold treatment. The change may be an indication that the calreticulin interacted with some membrane-protein complex. An over-expression of calreticulin also induces a greater increase of endogenous ABA. Calreticulin seems to be involved in the stress-signaling transduction system since it is phosphorylated strongly in cold treatment and $Ca^{2+}$ affects its phosphorylation. The activity of 47-kDa protein kinase is cold-induced and dependent on calreticulin. This suggests that an adaptation mechanism of rice mediated by calreticulin and CDPKs may exist to respond to cold treatment and that they may also be associated with the development of tolerance to various stresses.

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## REFERENCES

1) Monroy A. F., Dhindsa R. S., *Plant Cell*, **7**, 321—331 (1995).
2) Komatsu S., Kato A., *Phytochemistry*, **45**, 1329—1335 (1997).
3) Komatsu S., Karibe H., Hamada T., Rakwal R., *Theor. Appl. Genet.*, **98**, 1304—1310 (1999).
4) Milner R. E., Baksh S., Shemanko C., Carpenter M. R., Smillie L., Vance J. E., Opas M., Michalak M., *J. Biol. Chem.*, **266**, 7155—7165 (1991).
5) Saito Y., Ihara Y., Leach, M. R., Cohen-Doyle M. F., Williams D. B., *EMBO J.,* **18**, 6718—6729 (1999).
6) Li W. G., Komatsu S., *Theor. Appl. Genet.*, **101**, 355—363 (2000).
7) Knight H., Trewavas A. J., Knight M. R., *Plant Cell*, **8**, 489—503 (1996).
8) Knight M. R., Campbell A. K., Smith S. M. Trewavas A. J., *Nature* (London), **352**, 524—526 (1991).
9) Knight H., Trewavas A. J., Knight M. R., *Plant J.*, **12**, 1067—1078 (1997).
10) Denecke J., Carlsson L. E., Vidal S., Hoglund A. S., Ek B., van Zeijl M. J., Sinjorgo K. M., Palva E. T., *Plant Cell*, **7**, 391—406 (1995).
11) Chen T. H. H., Gusta L. V., *Plant Physiol.*, **73**, 71—75 (1983).
12) Thomashow M. F., "Arabidopsis," ed. by Meyarowitz E., Somerville C., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1994, pp. 807—834.
13) Levitt J., "Physiological Ecology: A series of Monographs, Texts and Treatises," Vol. 1, ed. by Kozlowsky T., Academic Press, New York, 1980, pp. 23—64.
14) Koga-Ban Y., Abe M., Kitagawa Y., *Plant Cell Physiol.*, **32**, 901—905 (1991).
15) Komatsu S., Masuda T., Abe K., *Plant Cell Physiol.*, **37**, 748—753 (1996).
16) Li Z., Komatsu S., *Eur. J. Biochem.*, **267**, 737—745 (2000).
17) Hood E. E., Helmer G. L., Fraley R. T., Chilton M. D., *J. Bacteriol.*, **168**, 1291—1301 (1986).
18) Chilton M. D., Currier T. C., Farrand S. K., Bendich A. J., Gordon M. P., Nester E. W., *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 3672—3676 (1974).
19) Hiei Y., Ohta S., Komari T., Kumashiro T., *Plant J.*, **6**, 271—282 (1994).
20) Komatsu S., Hirano H., *Plant Sci.*, **94**, 127—137 (1993).
21) Dedhar S., Rennie P. S., Shago M., Hagesteijn C. Y., Yang H., Filmus J., Hawley R. G., Bruchovsky N., Cheng H., Matusik R. J., *Nature* (London), **367**, 480—483 (1994).

22) Corbett E. F., Oikawa K., Francois P., Tessier D. C., Kay C., Bergeron J. J., Thomas D. Y., Krause K. H., Michalak M., *J. Biol. Chem.*, **274**, 6203—6211 (1999).
23) Willmitzer L., *Curr. Opin. Biotechnol.*, **7**, 161—167 (1996).
24) Crofts A., Leborgne-Castel N., Pesca M., Vitale A., Denecke J., *Plant Cell*, **10**, 813—824 (1998).
25) Li X., Su R. T., Hsu H. P., Sze H., *Plant Cell*, **10**, 119—130 (1998).
26) Chen H. H., Li P., Brenner M., *Plant Physiol.*, **71**, 362—365 (1983).
27) Savoure A., Hua X. J., Bertauche N., Van Montagu M., Verbruggen N., *Mol. Gen. Genet.*, **254**, 104—109 (1997).
28) Ishitani M., Xiong L., Stevenson B., Zhu J. K., *Plant Cell*, **9**, 1935—1949 (1997).
29) Droillard M. J., Guclu J., Le Caer J. P., Mathieu Y., Guern J., Lauriere C., *Planta*, **202**, 341—348 (1997).
30) Baldan B., Navazio L., Friso A., Mariani P., Meggio F., *Biochem. Biophys. Res. Commun.*, **221**, 498—502 (1996).
31) Mery L., Mesaeli N., Michalak M., Opas M., Lew D. P., Krause K. H., *J. Biol. Chem.*, **271**, 9332—9339 (1996).