

Over-expression of calcium-dependent protein kinase 13 and calreticulin interacting protein 1 confers cold tolerance on rice plants

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Abstract Calcium is a ubiquitous signaling molecule and changes in cytosolic calcium concentration are involved in plant responses to various stimuli. The rice calcium-dependent protein kinase 13 (CDPK13) and calreticulin interacting protein 1 (CRTintP1) have previously been reported to be involved in cold stress response in rice. In this study, rice lines transformed with sense CDPK13 or CRTintP1 constructs were produced and used to investigate the function of these proteins. When the plants were incubated at 5°C for 3 days, leaf blades of both the sense transgenic and vector control rice plants became wilted and curled. When the plants were transferred back to non-stress conditions after cold treatment, the leaf blades died, but the sheaths remained green in the sense transgenic rice plants. Expression of CDPK13 or CRTintP1 was further examined in several rice varieties including cold-tolerant rice varieties. Accumulation of these proteins in the cold-tolerant rice variety was higher than that in rice varieties that are intermediate in their cold tolerance. To examine whether over-expression of CDPK13 and CRTintP1 would have any effect on the proteins or

not, sense transgenic rice plants were analyzed using proteomics. The 2D-PAGE profiles of proteins from the vector control were compared with those of the sense transgenic rice plants. Two of the proteins that differed between these lines were calreticulins. The results suggest that CDPK13, calreticulin and CRTintP1 might be important signaling components for response to cold stress in rice.

Keywords Calreticulin · CDPK13 · CRTintP1 · Cold · Rice

Abbreviations

CDPK13 Calcium-dependent protein kinase 13
CRTintP1 Calreticulin binding protein 1

Introduction

Calcium has been recognized as an essential second messenger playing an important role in regulating a wide range of developmental, physiological and fundamental cellular processes in plants and animals (Clapham 1995; Corbett and Michalak 2000). A combination of changes in all calcium parameters produced by a particular signal is referred to as a calcium signature (Scrase-Field and Knight 2003). Although such calcium signatures may partially explain the specificity of cellular responses triggered by a particular stimulus, the molecules that “sense” and “interpret” the calcium signals provide additional specificity to the coupling of calcium parameters to cellular responses (McAinsh et al. 1992). Several families of calcium sensors have been identified in higher plants and are broadly divided into four major classes: calmodulin, calmodulin-like

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and other EF hand-containing calcium-binding proteins, calcium-regulated protein kinase and calcium-binding proteins without an EF-hand motif such as calreticulin (Zielinski 1998; Harmon et al. 2000; Crofts and Denecke 1998; Llewelyn et al. 1998).

Calreticulin is a major calcium-sequestering protein found in the lumen of the endoplasmic reticulum of a wide variety of eukaryotic cells, including those of higher plants (Corbett et al. 1999; Opas et al. 1996). In plants, calreticulin homologues have been purified and sequenced from spinach (Menegazzi et al. 1993), tobacco (Denecke et al. 1995), pea (Hassan et al. 1995), maize (Kwiatkowski et al. 1995) and rice (Komatsu et al. 1996; Li and Komatsu 2000). Abundant accumulation of calreticulin has been observed during callus regeneration in rice (Komatsu et al. 1996), during tobacco germination (Denecke et al. 1995), and in most plant tissues such as roots, young leaves (Menegazzi et al. 1993), and floral tissues of *Arabidopsis* (Nelson et al. 1997).

In rice, calreticulin has been identified as a calcium-binding phosphorylated protein that appears to be associated with the regeneration of cultured rice cells (Komatsu et al. 1996). Calreticulin is also present in rice suspension culture cells, and is developmentally regulated during regeneration (Li and Komatsu 2000). The role of calreticulin as a stress-induced molecular chaperone protein of the endoplasmic reticulum is becoming more apparent (Chapman et al. 1998). Rice calreticulin has been shown to be involved and phosphorylated in the signaling pathway leading to the cold stress response (Li et al. 2003; Khan et al. 2005). To investigate the biological role of calreticulin in rice, calreticulin interacting protein (CRTintP1) was isolated by a yeast two-hybrid interaction-cloning system (Sharma et al. 2004). Sharma et al. (2004) reported that co-immunoprecipitation using anti-calreticulin antibody confirmed the existence of the calreticulin-CRTintP1 complex in vivo in the cold stressed leaf tissue.

On the other hand, calcium-dependent protein kinase (CDPK) is the main calcium-regulated serine/threonine protein kinase in plants, and their genes are encoded by a multigene family (Hrabak 2004). CDPKs are important components in signal transduction, but the precise role of each CDPK is still largely unknown. Yang et al. (2003) reported that CDPK13 in rice, which is OsCPK7 (Asano et al. 2005), was an important signaling component in rice seedlings under cold stress condition and in response to gibberellin (GA). Furthermore, CDPK13 antisense transgenic rice were shorter than the vector control, and CDPK13 sense transgenic rice had higher recovery

rates after cold stress than the vector control (Abbasi et al. 2004).

Shen et al. (2003) reported that calreticulin was also an important component in the GA signaling pathway that regulates leaf-sheath elongation in rice seedling. This report indicated that calreticulin as well as CDPK13 may be an important signaling component in rice seedling under cold conditions and in response to GA. In addition, Li et al. (2003) has reported a possible interaction between calreticulin and CDPK. However, very little is known about the relationship among CDPK13, calreticulin and CRTintP1 under cold conditions. In this study, rice cultivars transformed with sense CDPK13 or CRTintP1 constructs were produced and were used for investigating the relationship among CDPK13, calreticulin and CRTintP1. The function of these proteins was characterized using proteome analysis.

Materials and methods

Construction of sense- and antisense-CDPK13, calreticulin and CRTintP1 transgenic rice plants

For CDPK13, calreticulin and CRTintP1 over expression and antisense transgenic rice, the full-length CDPK13 (Yang et al. 2003), calreticulin (Li and Komatsu 2000) and CRTintP1 (Sharma et al. 2004) cDNA sequences in the pBluescript SK+ plasmids were amplified by PCR using primer pairs. The resulting PCR products were digested, purified, and ligated between the 35SCaMV promoter and nopaline synthase terminator in the binary vector pIG121-Hm by replacing the GUS coding region (Ohta et al. 1990). The pIG121-Hm/CDPK13, calreticulin or CRTintP1 constructs were confirmed by restriction mapping and sequencing. The pIG121-Hm/CDPK13, calreticulin or CRTintP1 plasmids and vector control were then transferred into *Agrobacterium tumefaciens* strain EHA105 (Hood et al. 1986) and transformed into rice (*Oryza sativa* cv. Nipponbare, Kitaake or Basmati 370) (Toki et al. 2006). Transgenic rice plants were selected on medium-containing hygromycin. The hygromycin-resistant rice plants were transplanted to soil and grown to maturity at 30°C in 16 h light/8 h dark cycle in an isolating greenhouse.

Preparation of crude protein extract and cytosolic fraction

The following procedures were carried out at 4°C. For protein extraction, leaf sheaths (200 mg) were

homogenized in a mortar with a pestle in a 300 μ l extraction buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1 mM ethylene glycol-bis([beta]-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), 5 μ M sodium vanadate and 1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 20,000g for 5 min and the supernatant was used as the crude protein extract.

Leaf sheaths (200 mg) were homogenized in a mortar with a pestle in a 300 μ l extraction buffer containing 20 mM Tris–HCl (pH 7.5), 0.25 M sucrose, 10 mM EGTA, 1 mM dithiothreitol (DTT) and 1 mM PMSF. The homogenates were centrifuged at 3,000g for 5 min. The supernatants were centrifuged at 274,000g for 15 min and the cytosolic fraction was obtained by collection of the supernatant (Komatsu and Hirano 1993).

Gel electrophoresis

Samples (200 μ g, 100 μ l) solubilized with lysis buffer containing 8 M urea, 2% Triton X-100, 2% ampholine (pH 3.5–10) and 10% polyvinylpyrrolidone-40 (O'Farrell 1975) were separated in the first dimension by IEF tube gel and in the second dimension by SDS-PAGE (O'Farrell 1975). IEF tube gel solution consisted of 8 M urea, 3.5% acrylamide, 2% NP-40, 2% ampholines (pH 3.5–10.0 and pH 5.0–8.0), ammonium persulfate and TEMED. Electrophoresis was carried out at 200 V for 30 min, followed by 400 V for 16 h and 600 V for 1 h. After IEF, SDS-PAGE in the second dimension was performed using 15% polyacrylamide gel. The gels were stained with silver or Coomassie brilliant blue R-250 (CBB), and the image analysis was performed. Images of 2D polyacrylamide gel electrophoresis (2D-PAGE) were evaluated automatically using ImageMaster 2D Elite software (GE Healthcare, Piscataway, NJ, USA). The pI and Mr of each protein were determined using 2D-PAGE markers (Bio-Rad, Hercules, CA, USA).

Amino acid sequence analysis

Following separation by 2D-PAGE, the peptides were electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Pall, Port Washington, NY, USA) using a semidry transfer blotter (Nippon Eido, Tokyo, Japan), and detected by CBB staining. The stained protein spots were excised from the PVDF membrane and applied to a gas-phase protein sequencer Procise 494 (Applied Biosystems, Foster City, CA, USA). The amino acid sequences obtained were compared with those of known proteins in the Swiss-Prot, PIR,

Genpept and PDB databases with Web-accessible search program FastA.

Electrospray ionization quadrupole time of flight mass spectrometric analysis

The CBB stained protein spots were excised from gels and destained with 50 mM NH_4HCO_3 in 50% methanol at 40°C for 15 min. Proteins were reduced with 10 mM DTT in 100 mM NH_4HCO_3 at 50°C for 1 h and incubated with 40 mM iodoacetamide in 100 mM NH_4HCO_3 for 30 min. The gel pieces were minced and allowed to dry and then rehydrated in 10 mM Tris–HCl (pH 8.5) with 1 pmol trypsin at 37°C for 10 h. The digested peptides were extracted from the gel slices with 0.1% trifluoroacetic acid (TFA) in 50% acetonitrile/water for three times. Electrospray ionization (ESI)-MS/MS was carried out with a hybrid quadrupole orthogonal acceleration tandem mass spectrometer (Q-TOF; Micromass, Manchester, UK) connected with LC (Waters, Milford, MA, USA). MS/MS data were processed with a maximum entropy data enhancement program MaxEnt 3TM (Micromass). The resultant spectra were interpreted with SeqMS, software aids for de novo sequencing by MS/MS. The sequence tags obtained were also used for the homology search in the database with Mascot software (Matrix Science Ltd, London, UK).

Western blot analysis

Crude extracted proteins were subjected to SDS-PAGE and followed by Western blot analysis with an antibody or CBB staining. For separation of proteins by SDS-PAGE, 15% polyacrylamide gel was used, and proteins were electroblotted onto a PVDF membrane. The PVDF membranes were treated with anti-CDPK13 antibody (Abbasi et al. 2004), anti-calreticulin antibody (Komatsu et al. 1996) or anti-CRTintP1 antibody (Sharma et al. 2004), and antigen–antibody complexes were detected with enhanced chemiluminescence using ECLTM-Plus kit (GE Healthcare).

In-gel protein kinase assay

Cytosolic fraction was separated by 15% SDS-polyacrylamide gel containing 2 mg/ml histone III-S as a substrate for CDPK. For determining kinase activity, the gels were washed with a buffer containing 50 mM Tris–HCl (pH 8.0) and 20% 2-propanol for 1 h to remove SDS, and then with a buffer containing 50 mM Tris–HCl (pH 8.0) and 5 mM 2-mercaptoethanol (Buffer A) for 1 h. The proteins were denatured in

Buffer A containing 6 M guanidine-HCl for 1 h and renatured in Buffer A containing 0.04% Tween-40 at 4°C overnight. The gels were equilibrated in a buffer containing 40 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 2 mM DTT and 0.2 mM CaCl₂ for 30 min (Komatsu et al. 1993). The reactions were initiated by the addition of 50 μM [γ -³²P]ATP (GE Healthcare) and incubated at 30°C for 30 min. The reactions were stopped by washing extensively in 5% trichloroacetic acid and 1% sodium pyrophosphate. The gels were stained with CBB, destained, dried and exposed to X-ray film (Fuji, Tokyo, Japan) at -80°C for 1 day.

In vitro protein phosphorylation

Five micro liter crude protein extract was incubated in a 25 μl reaction mixture containing 20 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 0.2 mM CaCl₂ and 39 μM [γ -³²P]ATP (Komatsu et al. 1996). The reaction mixture was incubated for 10 min at 30°C and terminated by the addition of a lysis buffer (O'Farrell 1975). Denatured proteins were subjected to IEF in tube gels and then SDS-PAGE was carried out. The gels were stained with silver staining kit (Bio-Rad) or CBB, and exposed to X-ray film at -80°C for 5 days.

Results

Construction of sense- and antisense-CDPK13, calreticulin and CRTintP1 transgenic rice plants

To elucidate the role of calcium signaling and cold tolerance in rice, CDPK13, calreticulin and CRTintP1 transgenic rice plants containing sense and antisense constructs were generated. CDPK13 and calreticulin transgenic rice have been shown to respond to cold stress in our previous work (Li et al. 2003; Abbasi et al. 2004). In this study, sense CRTintP1 transgenic rice was also constructed, to investigate the relationship among CDPK13, calreticulin and CRTintP1. Furthermore, the sense transgenic rice was constructed in cold tolerance cultivar Kitaake, cold sensitive cultivar Basmati 370 and the intermediately cold-tolerant cultivar Nipponbare. Homozygous transgenic rice plants (T3) were used in this study. Ten lines were obtained for each sense transgenic rice plants. On the other hand, for antisense CDPK13 transgenic rice plant, three lines were obtained, and only one line for antisense CRTintP1 transgenic rice plant. They were selected for presented experiment by Western blot with anti-CDPK13 antibody (Abbasi et al. 2004), Anti-calreticulin antibody (Komatsu et al. 1996) or

anti-CRTintP1 antibody (Sharma et al. 2004). Each three lines were used for the following experiments.

Protein kinase activity and protein phosphorylation in the sense- and antisense-CDPK13 transgenic rice plants

The protein kinase activities in the *CDPK13* transgenic rice plants were investigated following an in-gel kinase assay. The activities of 60 and 70 kDa protein kinases were detected in the *CDPK13* sense and vector control plants (Fig. 1a). In contrast, the kinase activity of only

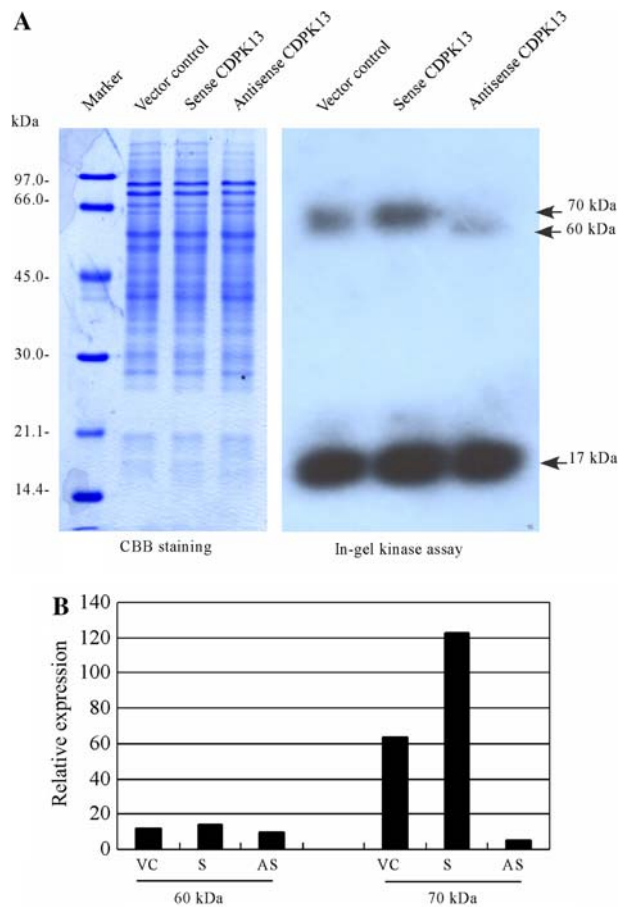


Fig. 1 Protein kinase activity in the *OsCDPK13* transgenic rice plants. **a** The cytosolic proteins from leaf sheaths of *OsCDPK13* transgenic rice seedlings containing sense and antisense constructs were separated by SDS-polyacrylamide containing 2 mg/ml histone III-S as a substrate for CDPK. The in-gel kinase assay was performed in the presence of 0.2 mM CaCl₂. The right plate shows protein kinase activity detected on X-ray film and the left plate shows CBB staining. A typical result is shown from three independent experiments. Arrows indicate the positions of 70, 60 and 17 kDa kinases. **b** Histogram shows the mean changes of protein kinase activities in cytosolic proteins of leaf sheath in vector control (VC), sense CDPK13 (S) and antisense CDPK13 (AS) transgenic rice. The experiment was repeated for three times and the means of three replications are shown

a 60 kDa protein was detected in the *CDPK13* anti-sense transgenic plants and the activity of 70 kDa protein kinase was not observed (Fig. 1b). This result indicates that the 70 kDa protein kinase is CDPK13. Another kinase activity at 17 kDa was observed in all plants, which might be attributed to the nucleoside diphosphate kinase as previously reported (Hamada et al. 1999).

To evaluate the changes in phosphorylation status of the proteins in *CDPK13* transgenic rice plants containing the sense constructs, an in vitro phosphorylation of the proteins was carried out. The phosphorylation status of the proteins was detected by labeling the proteins with [γ - 32 P]ATP. After in vitro phosphorylation, the proteins were separated by 2D-PAGE and visualized by silver or CBB staining and exposing the 2D-gels on X-ray film (Fig. 2). Seven phosphorylated proteins were compared in sense *CDPK13* transgenic rice and vector control plant. The phosphorylation status of two proteins having molecular weights and pI values 56.0/5.2 (spot number 1) and 32.1/7.0 (spot number 2), respectively, were enhanced in sense *CDPK13* transgenic rice (Fig. 2).

To substantiate further this result, attempts were made to identify these proteins by ESI-MS/MS. 32 P-labelled phosphoproteins resolved by 2D-PAGE were excised and digested in-gel with trypsin. Several tryptic peptides of the proteins were analyzed by ESI-MS/MS, yielding MS/MS fragment ions that were sufficient for acquiring the sequence tags of the peptides. Identification of the proteins from the sequence tag revealed that these proteins were calreticulin (spot number 1) and GTPase Ras 2p (spot number 2). Calreticulin and GTPase Ras are all involved in regulating intracellular calcium signaling. Sense-*CDPK13* transgenic rice displaying enhanced phosphorylation of calreticulin and GTPase Ras indicates that these proteins are phosphorylated downstream of CDPK13, and the phosphorylation is probably an important mechanism in decoding intracellular calcium signaling.

Expression of calreticulin and CRTintP1 proteins in the sense-*calreticulin* and *CRTintP1* transgenic plants

CRTintP1 was identified as a calreticulin-interacting protein by yeast two-hybrid systems and was found bound to calreticulin using immunoprecipitation system (Sharma et al. 2004). To elucidate the roles of calreticulin and CRTintP1, the two protein levels were analyzed in sense-*calreticulin* and *CRTintP1* transgenic rice. Accumulation of calreticulin protein and CRTintP1 protein in the sense *calreticulin* and *CRTintP1* transgenic

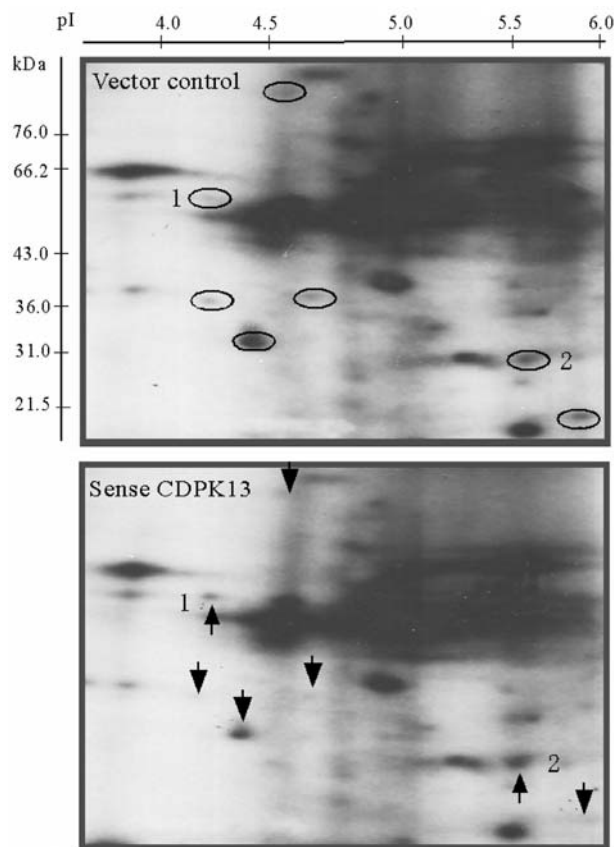


Fig. 2 Protein phosphorylation in the *OsCDPK13* transgenic rice plants. Crude proteins were extracted from leaf sheaths of *OsCDPK13* transgenic rice seedlings containing sense construct and incubated with the reaction mixture containing 0.2 mM CaCl_2 . After in vitro protein phosphorylation, phosphorylated proteins were separated by 2D-PAGE with IEF in the first dimension and SDS-PAGE in the second dimension. The proteins were detected by silver staining and exposed to X-ray film. A typical result is shown from five independent experiments. Arrows indicate the positions of phosphoproteins whose abundance changed in the sense transgenic plants, while circles represent the same proteins in vector control. Numbers 1 and 2 show the phosphorylated proteins in the sense *CDPK13* transgenic rice as compared with vector-control transgenic rice

rice was higher than those in vector control transgenic rice, respectively (Fig. 3). Calreticulin protein in sense *CRTintP1* transgenic rice and *CRTintP1* in sense *calreticulin* transgenic rice were also higher than that in the vector-control transgenic rice. These data demonstrate the interaction of calreticulin with *CRTintP1* in vivo, which is in agreement with the observations obtained from the yeast two-hybrid analysis (Sharma et al. 2004).

CDPK13, calreticulin and *CRTintP1* expression in cold-tolerant rice varieties

CDPK13, calreticulin and *CRTintP1* were further examined in various rice varieties, including the

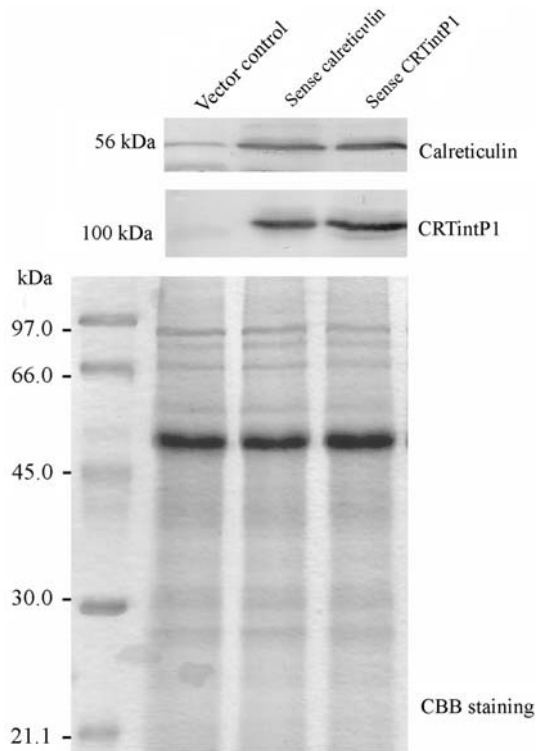


Fig. 3 Differences in protein accumulation of calreticulin and CRTintP1 in the sense calreticulin and CRTintP1 transgenic rice. Crude proteins were extracted from sense calreticulin and CRTintP1 transgenic rice grown in isolating greenhouse. Each protein extract was separated by SDS-PAGE, blotted onto a PVDF membrane and reacted with anti-calreticulin or CRTintP1 antibody. CBB staining was used as loading control. The experiment was repeated three times

cold-tolerant rice varieties, “Arroz da Terra” and “Ouu-choku 376” and the intermediately cold-tolerant varieties “Fukuhibiki”, “Akitakomachi” and “Nipponbare” (Ogawa and Tarashima 2001; Miura 2003) (Fig. 4). Accumulation of CRTintP1 in cold tolerant rice varieties and Fukuhibiki was clearly higher than that in rice varieties that are intermediate in their cold tolerance. Accumulation of calreticulin in cold-tolerant rice varieties was also higher than in these three rice varieties. On the other hand, in the case of CDPK13, Ouu-choku 376 has highest protein level. CDPK13 level in Arroz da Terra was similar to that in Fukuhibiki and Akitakomachi and it was very low in Nipponbare (Fig. 4). Abbasi et al. (2004) reported that accumulation of CDPK13 was not detected in cold-sensitive rice variety. Accumulation of calreticulin and CRTintP1 was not detected in cold-sensitive rice varieties (data not shown). A physiological role for these proteins in cold tolerance is supported by the phenotype of transgenic rice lines with enhanced expression of these proteins.

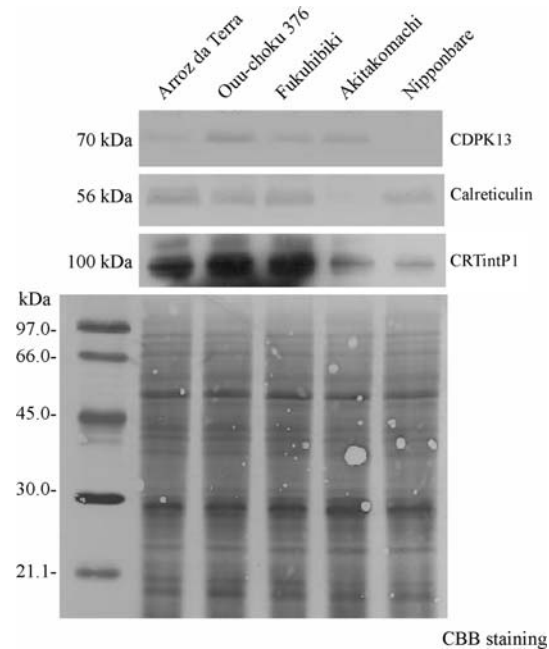


Fig. 4 Differences in protein accumulation of CDPK13, calreticulin and CRTintP1 among cultivars. Crude proteins were extracted from rice cv. “Arroz da Terra”, “Ouu-choku 376”, “Fukuhibiki”, “Akitakomachi” and “Nipponbare” grown in growth chamber. Each protein extract was separated by SDS-PAGE, blotted onto PVDF membrane and reacted with anti-CDPK13, calreticulin or CRTintP1 antibody. CBB staining was used as a loading control. The experiment was repeated five times

Sense transgenic rice lines are tolerant to cold stress

To elucidate the role of CDPK13 and CRTintP1 in stress tolerance, additional sense transgenic rice lines were constructed with cold sensitive cultivar Basmati 370 and with cold tolerant cultivar Kitaake. When seeds of vector-control transgenic rice were incubated at 15°C for 17 days, germination percentages were 45 and 13% in “Nipponbare” and “Basmati 370”, respectively (Figs. 5, 6a). However, in the sense-CDPK13 transgenic rice, germination percentages raised to 72 and 83% in “Nipponbare” and “Basmati 370”, respectively (Fig. 5a). In the sense-CRTintP1 transgenic rice, the germination percentages were 92 and 88% in “Nipponbare” and “Basmati”, respectively (Fig. 6a).

When 2-week-old rice seedling plants were incubated at 5°C for 3 days, the leaf blades of rice plants became wilted and curled. When plants were transferred back to non-stress conditions after cold treatment, the leaf blades died, but the sheaths remained green in the tolerant rice plant (Abbasi et al. 2004). In this study, when the vector-control transgenic rice plant of 2-week-old seedlings were incubated at 5°C for 3 days and transferred back to non-stress conditions, the ratio of survival was 18 and 19% in “Nipponbare”

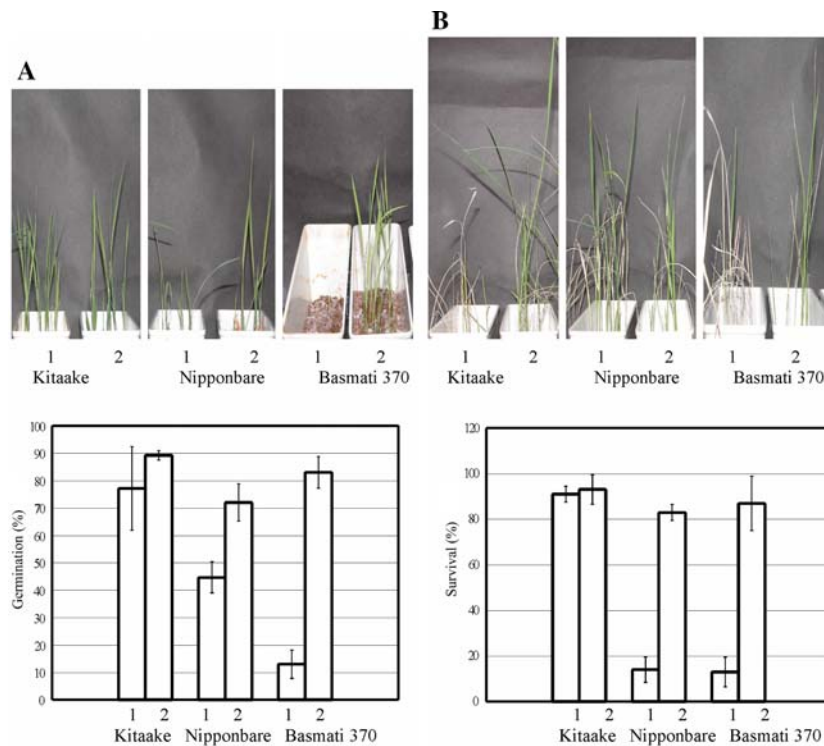


Fig. 5 Effect on cold tolerance of over-expressed CDPK13. Sense CDPK13 transgenic rice (cv. “Nipponbare”, “Kitaake” and “Basmati 370”) were used. **a** Rice seeds were directly planted in the soil, and treated at 15°C for 17 days. Cold tolerance was estimated as the percentage of plants germinating under non-stress conditions. **b** Rice seeds were germinated at 25°C for 3 days, and

planted in the soil, and then grown at 25°C. Two-week-old rice seedlings were treated at 5°C for 3 days, and cold tolerance was estimated as the percentage of plants surviving after 14 days recovery under non-stress conditions. “1” shows the vector control and “2” shows sense-CDPK13 transgenic rice. Ten individual plants of each transgenic line were tested. *Error bars* indicate SE

and “Basmati 370”, respectively (Figs. 5, 6b). However, in the sense-CDPK13 transgenic rice, the ratio of survival was 81 and 83% in “Nipponbare” and “Basmati 370”, respectively (Fig. 5b). In the sense-CRTintP1 transgenic rice, the ratio of survival was 92 and 80% in “Nipponbare” and “Basmati”, respectively (Fig. 6b). Taken together, the results show that over expression of CDPK13 and CRTintP1 can protect plants from cold damage and lead to enhanced levels of germination (15°C) and seedling survival (5°C). These results show that CDPK13 and CRTintP1 are involved in the response to cold stress in rice.

The effects of over-expression of CDPK13 and CRTintP1 in rice

To examine whether the over-expression of CDPK13 and CRTintP1 would have any effect on patterns of protein accumulation or not, sense transgenic rice plants were analyzed using proteomics technique. Two-week-old seedlings of sense CDPK13 and CRTintP1 transgenic rice were used. Crude proteins were extracted from leaf sheaths, separated by 2D-PAGE, and stained

by CBB (Fig. 7). Proteins whose abundance changed in transgenic rice leaf sheaths were excised from 2D-gels and identified by protein sequencer or MS (Table 1). Out of 456 proteins, 6 proteins accumulated in the cold tolerant rice variety “Kitaake”, but they did not accumulate in the cold-sensitive rice variety “Basmati 370” in the vector control. In sense-transgenic rice, the six proteins were up regulated as compared with those in vector control. This result shows that accumulation of these six proteins is related to cold signaling.

Two of the six proteins were calreticulin (spots 1 and 2) that must be related to the actions of CDPK13 and CRTintP1. Four proteins, fructokinase (spot 5), cytoplasmic malate dehydrogenase (spot 6), initiation factor 4A (spot 3) and alpha-tubulin (spot 4), were also up regulated in sense CDPK13 and CRTintP1 transgenic rice.

Discussion

The mechanisms by which low-temperature signals are perceived by plants and transduced into biochemical responses are poorly understood. Plants exhibit a

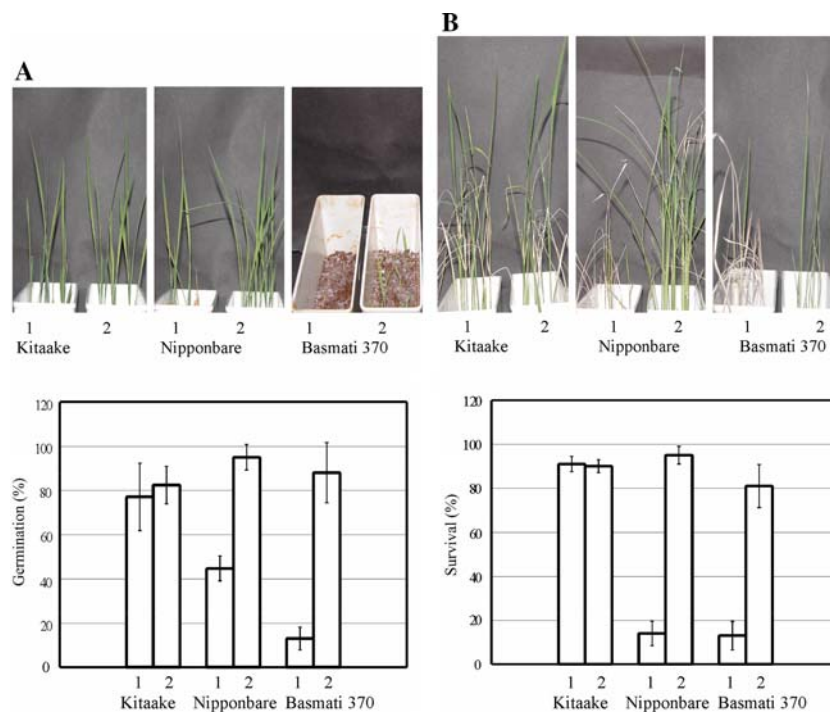


Fig. 6 Effect on cold tolerance of over-expressed CRTintP1. Sense CRTintP1 transgenic rice (cv. “Nipponbare”, “Kitaake” and “Basmati 370”) were used. **a** Rice seeds were directly planted in the soil, and treated at 15°C for 17 days. Cold tolerance was estimated as the percentage of plants germinating under non-stress conditions. **b** Rice seeds were germinated at 25°C for 3 days, and planted in the soil, and then grown at 25°C. Two-

week-old rice seedlings were treated at 5°C for 3 days, and cold tolerance was estimated as the percentage of plants surviving after 14 days recovery under non-stress conditions. “1” shows the vector control and “2” shows sense-CRTintP1 transgenic rice. Ten individual plants of each transgenic line were tested. Error bars indicate SE. Vector controls are the same as those of Fig. 5

biphasic response to low temperatures: an early set of responses that includes a shift in membrane fluidity and cold-induced calcium influx, and a secondary set of responses in which calcium- and cold-regulated protein kinases and phosphatase are believed to be involved (Monroy et al. 1998). To investigate the relationship among CDPK13, calreticulin and CRTintP1, rice transformed with sense CDPK13, CRTintP1 and calreticulin constructs were produced and used.

CDPK13 has a high level of identity with three other rice CDPKs, namely CDPK1, CDPK11, and CDPK12 (Asano et al. 2005). In rice, at least two isoforms of calreticulin were evident based on the restriction enzyme pattern differences between the calreticulin clones (Li and Komatsu 2000). However, it was not known whether these increases in mRNA levels were accompanied by increases in protein levels and/or kinase/phosphorylation activity. In the sense CDPK13 transgenic rice, calreticulin was phosphorylated (Fig. 2). Additionally, CRTintP1 was accumulated in the sense calreticulin transgenic rice, and calreticulin was accumulated in the sense CRTintP1 transgenic rice (Fig. 3). Furthermore, Sharma et al. (2004) reported that CRTintP1 was identified as a calreticulin-interacting

protein by yeast two-hybrid system and was binding to calreticulin using immuno-precipitation system. These results indicate that CDPK13, calreticulin and CRTintP1 were related with each others.

Abbasi et al. (2004) reported that sense CDPK13 transgenic rice had higher recovery rates after cold stress than the vector control. Furthermore, rice cultivars transformed with sense CDPK13 and CRTintP1 were produced (Figs. 5, 6). Over expression of CDPK13 and CRTintP1 in the cold sensitive cultivar Basmati 370 could protect plants from cold damage. These results indicate that CDPK13 and CRTintP1 are involved in the response to cold stress in rice.

To analyze the steps downstream of CDPK13 and CRTintP1 which were induced by cold stress, proteomics technique was used (Fig. 7). Many proteins were changed under the cold stress (Yang et al. 2006), and we examined whether or not they are acting downstream of CDPK13 and CRTintP1. Cold stress leads to major alternations in carbohydrate metabolism (Thomashow et al. 1999; Wanner and Junttila 1999) and the sugar signaling pathways interact with stress pathways to modulate metabolism. The sugar status of plant cells is sensed by sensor proteins. The signal

Fig. 7 2D-PAGE pattern of leaf sheath proteins in transgenic rice. **a** Two-week-old sense transgenic rice cv. “Nipponbare” was used. Crude proteins were extracted from leaf sheaths, separated by 2D-PAGE, and stained by CBB. *Arrowheads* denote the positions of proteins up regulated in sense CDPK13 and CRTintP1 transgenic rice plants (cv. “Nipponbare”), while circles represent the same proteins in vector control. **b** Two-week-old sense transgenic rice cv. “Nipponbare”, “Kitaake” and “Basmati 370” were used. Only the protein spots detected on 2D-gels showed in **a** were picked up to show the changes of the proteins in sense transgenic rice. *Arrowheads* denote the positions of proteins up regulated in other transgenic rice as compared with vector control cv. “Nipponbare”

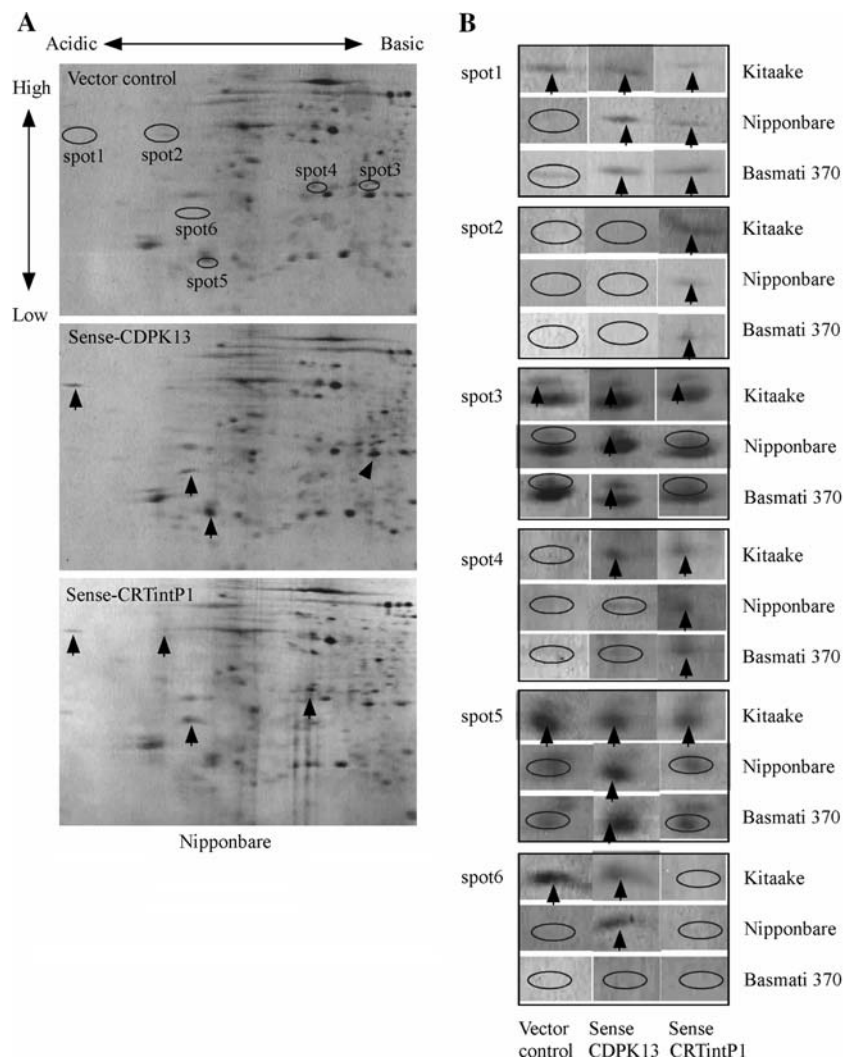


Table 1 Identification of proteins changed in CDPK13- and CRTintP1-sense transgenic plant

Spot no.	MW	pI	Homologous protein (homology or score)	Accession no.	ID
1	56	4.2	Calreticulin precursor (100)	Q9SP22	Ed (N-EVFFQEK)
2	56	4.5	Calreticulin precursor (100)	Q9SP22	Ed (N-EVFFQEK)
3	46	6.5	Initiation factor 4A (74)	AB046416	MS
4	46	6.0	Alpha-tubulin (80)	AK102560	MS
5	34	5.2	Fructokinase (92)	AY197773	MS
6	38	4.8	Cytoplasmic malate dehydrogenase (62)	AF353203	MS

MS, MALDI-TOF-MS; Ed, protein sequencer; N-, N-terminal amino acid sequences determined by Edman degradation

generated by signal transduction cascades, which could involve protein kinases, protein phosphatases and calcium, results in appropriate gene expression. Hexokinase-dependent and -independent pathways are involved in sugar sensing. Sucrose also acts a signal molecule as it affects the activity of a proton-sucrose symporter (Gupta and Kaur 2005). Fructokinase may represent an additional sensor that bypasses hexokinase phosphorylation especially when sucrose is dominant.

Cytoplasmic malate dehydrogenase reversibly catalyzes the incorporation of malate to oxaloacetate utilizing the NAD^+/NADH cofactor (Minarik et al. 2002). Maldonado et al. (2004) reported that the high cytoplasmic malate dehydrogenase enzyme activity and the strong stimulation of NADP-malic enzyme activity exhibited by CO_2 -treated fruit could be contributing factors in the maintenance of fruit energy metabolism, pH stability, and the promotion of synthesis of defense compounds that prevent or repair damage caused by

chilling temperature. Our study suggests that cytoplasmic malate dehydrogenase could serve to prevent or repair damage caused by cold tolerance in rice.

Vashisht and Tuteja (2005) reported cold stress-induced pea DNA helicase 47 is homologous to initiation factor 4A and is inhibited by DNA-interacting ligands. In this study, initiation factor 4A was also detected in cold tolerant transgenic rice. These results should help contribute to a better understanding of cold stress signaling and mechanisms of DNA unwinding in plants. Furthermore, Abdrakhamanova et al. (2003) reported that the appearance of cold-stable microtubules in wheat was accompanied by a reduced abundance of type TUA1/2 alpha-tubulin isotypes. Alpha-tubulin was also detected in cold tolerant transgenic rice. These results suggest a role of microtubule disassembly in the sensing of low-temperature stress.

Taken together, the results presented here suggest that calreticulin provides multiple functions in rice. The identification of calreticulin as a phosphoprotein during rice seedling germination suggests that its role might be catalyzed by phosphorylation in the cytoplasm. Although further work is required to clarify the details of CDPK13, calreticulin and CRTintP1 function, our results demonstrate that simple manipulation of CDPK13, calreticulin and CRTintP1 activity has great potential for the improvement of rice. The results suggest that CDPK13, calreticulin and CRTintP1 might be important signaling components for response to cold stress in rice.

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