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# Cold stress-induced calcium-dependent protein kinase(s) in rice (*Oryza sativa* L.) seedling stem tissues

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**Abstract** $Ca^{2+}$-dependent protein kinases (CDPKs) play an important role in plant signal transduction. Protein kinase(s) activities induced by 5°C cold stress in rice (*Oryza sativa* L.) seedlings were investigated in both leaf and stem tissues in an early (up to 45 min) and late (up to 12 h) response study. The leaf had 37-, 47- and 55-kDa protein kinase activities, and the stem had 37-, 47- and 55-kDa protein kinase activities. A 16-kDa protein showed constitutive kinase activity in the rice seedling leaf and stem. It was further identified that the 47-kDa protein kinase activity induced by cold in both the cytosolic and membrane fractions of the stem was strictly $Ca^{2+}$-dependent. This CDPK activitiy increased in the presence of the $Ca^{2+}$ ionophore A23187 in stem segments, whereas it was decreased by the $Ca^{2+}$ channel blocker, $LaCl_3$, and the $Ca^{2+}$ chelator, EGTA. The general protein kinase inhibitor, staurosporine, completely inhibited this CDPK activity in vitro, and both W7, a calmodulin antagonist, and H7, a protein kinase C inhibitor, could only partially decrease this activity. The protein phosphatase inhibitor, okadaic acid, increased CDPK activity. This CDPK activity was also induced by salt, drought stress and the phytohormone abscicic acid. Among the 18 rice varieties tested, this cold-induced 47-kDa CDPK activity was stronger in the cold-tolerant varieties than in the sensitive ones.

**Key words** Cold stress · $Ca^{2+}$-dependent protein kinase · In-gel phosphorylation assay · Rice

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## Introduction

Plants can respond to a variety of biotic and abiotic signals or factors that affect their growth and development. Although the responses to these signals and factors in plants have been extensively studied at physiological and biochemical levels, the perception and intracellular transmission mechanisms remain largely unknown. Under natural growth conditions, plants do encounter various stress conditions, such as drought, salinity, high or low temperatures, dehydration and osmosis, which have profound effects on their growth and development. Rice seedlings can respond to cold treatment with major changes in gene expression (Hahn and Walbot 1989). It is generally recognized that patterns of protein syntehsis and mRNA levels change when plants are exposed to cold (Thomashow 1990). Various stresses have been shown to influence protein phosphorylation (Anderberg and Walker-Simmons 1992; Garbarino et al. 1991). During early stages of cold acclimation, the phosphorylation of cellular proteins and activation of protein kinases has been detected (Holappa and Walker-Simmons 1995). After being subjected to cold stress cold-sensitive rice varieties showed a similar change in protein phosphorylation pattern in contrast to the cold-tolerant rice varieties (Komatsu and Kato 1997). Recently, it was reported that rice seedlings respond to a cold stress of 5°C by definite changes in protein phosphorylation (Komatsu et al. 1999).

Low-temperature treatment has been shown to induce an increase in cytosolic $Ca^{2+}$ levels (Knight et al. 1991, 1996), and $Ca^{2+}$ could play an essential role in the cold acclimation process (Knight et al. 1997; Monroy et al. 1993). $Ca^{2+}$ plays an important role in numerous physiological processes, and the presence of $Ca^{2+}$-regulated protein kinases will have wide implications in cellular signal transduction (Poovaiah and Reddy 1993). Sheen (1996) has shown the involvement of $Ca^{2+}$-dependent protein kinases (CDPKs) in stress signal transduction. CDPKs play a key role in stress and $Ca^{2+}$-mediated signal transduction. As a specific group of kinases that re-

quire only micromolar concentrations of $Ca^{2+}$ for their activity, CDPKs do not need calmodulin or phospholipids in plants and have been identified as being $Ca^{2+}$-dependent, calmodulin-independent serine/threonine kinases (Stone and Walker 1995; Poovaiah et al. 1997). Plant CDPKs have been shown to phosphorylate a number of exogenous substrates, including histone III-S (Polya et al. 1989). CDPKs exist as multiple isoforms (Verhey et al. 1993), and the hybridization and isolation of cDNA has further revealed that these enzymes are encoded by a multigene family (Harper et al. 1993; Kim et al. 1998). As for their localization, CDPKs are either membrane-associated (Klucis and Polya 1988; Ohto and Nakamura 1995) or cytosolic in nature (Polya et al. 1987; Komatsu and Hirano 1993). An in-gel phosphorylation assay revealed the presence of a 32-kDa CDPK during the regeneration of rice cultured suspension cells (Karibe and Komatsu 1998); $Ca^{2+}$- and phospholipid-dependent protein kinases in rice leaves had been identified in previous studies (Karibe and Komatsu 1997), although their functions still remain to be clarified. A 45-kDa CDPK which can phosphorylate histone III-S in-gel was found in rice leaves cultured in darkness in the presence of $Ca^{2+}$ and phosphatidylserine (Karibe et al. 1996).

In this study reported here, we investigated the presence of phosphorylation activity in rice seedling tissues after exposure to cold in early- and a late-response experiment, with the specific aim of identifying CDPK activity. The CDPK was characterized using protein kinase inhibitors, a calmodulin antagonist, a $Ca^{2+}$ chelator, a channel blocker and a $Ca^{2+}$ ionophore. The influence of certain stress factors, like drought, high salt and the phytohormone absicsic acid (ABA), were also examined for their effect on CDPK activity in rice seedlings.

## Materials and methods

### Plant materials

Rice (*Oryza sativa* L.) cvs. Nipponbare, Kitaibuki, IR36, Yukihikari, Sensyo, Akita 39, Akihikari, Akitairi, Reimei, Takaneminori, Hayakogane, Akitakomachi, Sasanishiki, Hayamasari, Yamahoushi, Motsuhomare, Hamaasahi and ARROZ seedlings were used in this experiment. The seedlings were grown for 2 weeks in a growth chamber after germination under fluorescent light (about 600 µmol $m^{-2}s^{-1}$, 12-h light/dark regime) at 25°C and 75% relative humidity. Fourteen-day-old rice seedlings and stem segments were subjected to cold stress at 5°C in a cold chamber under the same light and relative humidity conditions as normal growth conditions. Stem segments 3-cm-long were cut from seedlings and floated on pure water in petri dishes supplemented with 250 m*M* NaCl for salt stress or 50 µ*M* ABA for the phytohormone experiment, or directly in a growth chamber at 25°C until they had lost 50% fresh weight (for drought stress). Cultivar Nipponbare was used as the main material for this study. Rice seedling leaf, stem and stem segment tissues were homogenized after various treatments and analyzed as described below.

### Chemicals

Staurosporine, $Ca^{2+}$ ionophore A23187, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), $LaCl_3$ and ABA were purchased from Wako Pure Chemical Industries (Osaka). Histone III-S, myelin basic protein (MBP) and okadaic acid were from Sigma (St. Louis, Mo.). *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7) and 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H7) were from Seikagaku Kogyo Co Ltd (Tokyo).

### Preparation of cytosolic and membrane protein fractions

Rice seedling tissues (250 mg) were homogenized immediately with 500 µl (leaf) or 250 µl (stem) homogenization buffer containing 20 m*M* TRIS-HCl (pH 7.5), 0.25 *M* sucrose, 10 m*M* EGTA, 1 m*M* dithothreitol (DTT) and 1 m*M* phenylmethylsulfonyl fluoride (Komatsu and Hirano 1993). The homogenates were centrifuged at 800 *g* for 5 min, and the supernatants were centrifuged at 100000 *g* for 15 min. The cytosolic fraction was obtained by collection of the supernatant. The pellet was washed in 100 µl homogenization buffer by centrifugation at 100000 *g* for 15 min. The pellet was resuspended in 50 µl of membrane solubilizing buffer containing 1% Triton X-100, 20 m*M* TRIS-HCl (pH 7.5), 1 m*M* EDTA and 50 m*M* 2-mercaptoethanol and then solubilized on ice for 30 min. The membrane fraction was obtained by centrifugation at 100000 *g* for 7 min.

### In-gel phosphorylation assay

Protein extracts were separated on 17% sodium dodecyl sulfate (SDS)-polyacrylamide gels embedded with 2 mg/ml histone III-S or 0.5 mg/ml MBP as the substrate for the protein kinase in the separating gel. After electrophoresis, the SDS was removed by washing the gel for 1 h with a buffer containing 50 m*M* TRIS-HCl (pH 8.0), 20% 2-propanol, and then for 1 h with a 50 m*M* TRIS-HCl (pH 8.0) solution containing 5 m*M* 2-mercaptoethanol (Buffer A). The separated proteins were denaturated for 1 h in Buffer A containing 6 *M* guanidine-HCl, and then allowed to renature in Buffer A containing 0.04% (w/v) Tween-40 at 4°C for 16 h. The proteins were then reacted with 50 µ*M* γ-[$^{32}$P] ATP (0.45 MBq, Amersham, Buchinghamshire) for 30 min at room temperature in 40 m*M* TRIS-HCl (pH 8.0), 10 m*M* $MgCl_2$, 2 m*M* DTT and 0.2 m*M* $CaCl_2$. The reaction was stopped by extensive gel washing with 5% (w/v) trichloroacetic acid containing 1% potassium pyrophosphate. The gels were stained with Coomassie Brilliant blue R-250 (CBB), destained, dried and exposed to X-ray film (Kodak, Rochester, N.Y.).

## Results and discussion

### Rice seedlings respond to cold stress by early changes in protein kinase activities

CDPKs play an important role in plant signal transduction. In this study, we investigated the presence of phosphorylation activity in rice seedling tissues after exposure to cold in an early- and a late-response experiment, with the specific aim of identifying CDPK activities. In an early-response study lasting for 15–45 min, rice seedling tissues responded to cold treatment with the induction of protein kinase activities under both dark and light conditions. These changes were mainly observed in the cytosolic fractions in leaf tissues with no response being observed in the membrane fractions. Three protein kinase activities induced in leaf cytosolic fractions by the cold treatment were found under dark conditions at approximately molecular weights of 55 kDa, 47 kDa and

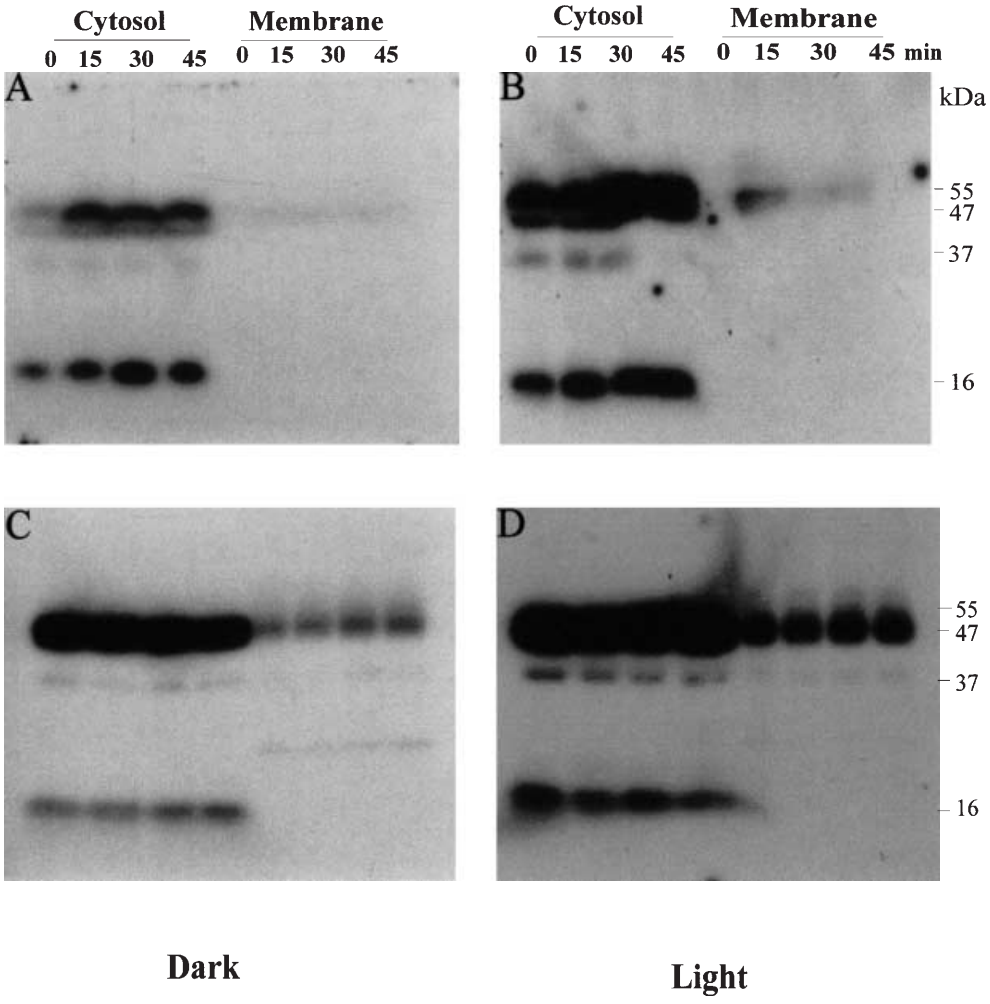


**Fig. 1A–D** Protein kinase activities induced by cold stress in cytosolic and membrane fractions of rice seedlings under dark or light conditions. Leaf (**A**, **B**) and stem (**C**, **D**) tissues were collected after cold treatment for 0, 15, 30 and 45 min. Activities were detected by autoradiography using histone III-S as the substrate for the in-gel phosphorylation assay

16 kDa, while a 37-kDa protein kinase activity was found only under light conditions (Fig. 1). The 16-kDa protein kinase activity in the leaf increased in a time-course manner related to length of cold stress under both dark and light conditions.

In stem tissues, at least four protein kinase activities were observed of which the 16- and 47-kDa protein kinases were common under both light and dark conditions. The 47-kDa protein kinase activity also existed in both the cytosolic and membrane fractions, remaining stable in the cytosol fraction but increasing in the membrane fraction under both dark and light conditions after cold treatment (Fig. 1). However, the 47-kDa activity induced by cold and dark conditions is clearly time-dependent under the short-time course experiment; in contrast, light conditions only slight increased that activity.

These results show that rice does respond to cold stress with the induction of protein kinase activity in rice seedling tissues, especially in the stem membrane fractions, in an increasing line as an early response after cold treatment at 5°C. The 16-kDa CDPK activity was found to be constitutively present in the rice seedling tissue cytosolic fractions examined. Low temperature is an important factor affecting plant growth and development, not only inducing cold acclimation but also resulting in some extensive changes involved in the signal transduction pathway mediated through calcium (Knight et al. 1996) and protein kinase (Sheen 1996).

## Cold-induced late-response protein kinase activities in rice seedlings

To determine the effect of long-time response to cold stress we subjected rice seedling tissues to a 3- to 12-h-long cold treatment under light conditions, except that the initial 3 h were under dark conditions. The changes in the protein kinase activity of the rice seedling leaf and stem tissues were quite similar to those observed during the short-time cold stress under light conditions, with similar amounts and sizes of protein kinase activities being induced by cold stress. Compared to the controls, which were carried out at the same time with cold treatment, an increasing level of 47-kDa protein kinase activity over time was induced in stem membrane fractions (Fig. 2); this was very similar to that observed during the short-time induction by cold (Fig. 1). The two protein kinase activities of 55 kDa and 47 kDa in the leaf cytosolic

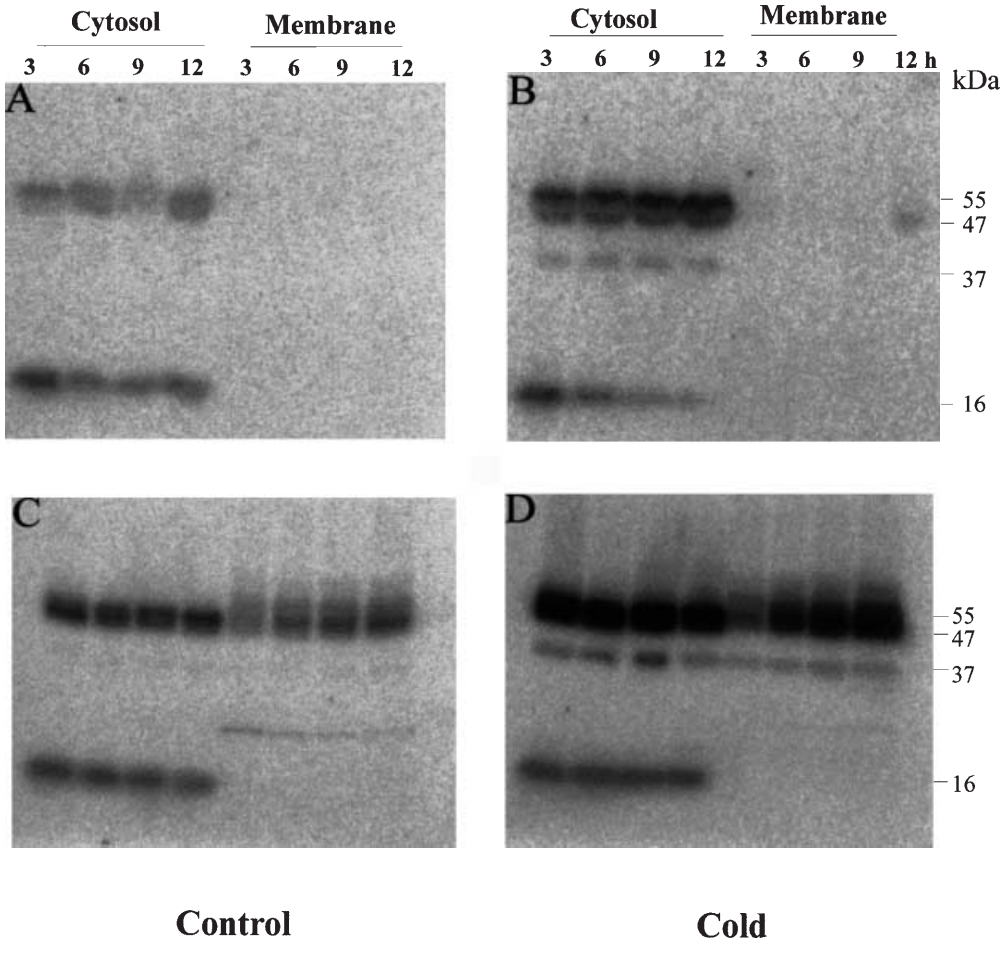


**Fig. 2A–D** Protein kinase activities induced by cold stress in cytosolic and membrane fractions of rice seedlings under light conditions. Leaf (**A**, **B**) and stem (**C**, **D**) tissues were collected after a cold stress treatment for 3, 6, 9 and 12 h (the initial 3 h were under dark conditions). Activities were detected by autoradiography using histone III-s as the substrate for the in-gel phosphorylation assay

fraction induced by cold showed only a slight increase during 3 the 12 h of cold treatment. The 47-kDa protein kinase activity in the stem membrane fraction was dramatically induced by cold stress, in a time-dependent manner, but it remained stable in the cytosolic fractions. These data demonstrate that rice seedlings can respond to low-temperature treatment continuously up to 12 h and that different CDPKs activities in different tissues are induced (leaf, stem). It is quite possible that numerous CDPKs exist in a plant as they are encoded by multigene families and show diversity in responsiveness to $Ca^{2+}$ levels, expression patterns, subcellular localization and physiological functions (Stone and Walker 1995; Hong et al. 1996).

## Identification of CDPK activity in rice seedling stem tissues

A 47-kDa protein kinase activity which showed a strong dependence on calcium in rice seedling stem tissues was studied in more detail. CDPK activities in the rice seedling stems were investigated during both early and late responses to cold stress (Fig. 3). The 47-kDa protein kinase activity was strongly induced only when $Ca^{2+}$ and histone III-S were the substrates for in-gel kinase assay. This activity increased in a time-dependent manner in the membrane fraction, however, it remained stable in the cytosolic fraction. The $Ca^{2+}$ chelator, EGTA, completely blocked this induction, whereas other treatments, without substrate or using MBP as the substrate, showed no significant effect on this activity. These results clearly indicate that the 47-kDa protein kinase is a CDPK that demonstrates strict $Ca^{2+}$ dependence in stem tissues, which is a characteristic of CDPK, in that it shows a high activity in the presence of $Ca^{2+}$. It was also shown that this CDPK has a slight auto-phosphorylation activity, however no differences were found either when using MBP as the substrate and without substrate. The 16-kDa protein appeared to show MBP-like kinase activity in both the cytosolic and membrane fractions. This 47-kDa CDPK was further characterized by liquid-phase isoelectric focusing using the Rotofor cell system (Bio-Rad, Alfred Nobel Drive Hercules, Calif.) and the pI was determined to be 6.25 (data not shown).

These experiments show that the 47-kDa protein kinase is a CDPK rather than a MBP-like kinase, although MBP-like kinase activity can be induced by low temperatures (Jonak et al. 1996; Mizoguchi et al. 1996). This CDPK was clearly induced in an increasing manner in the membrane fraction of stem tissue after cold stress. It has been shown that low temperature is involved in

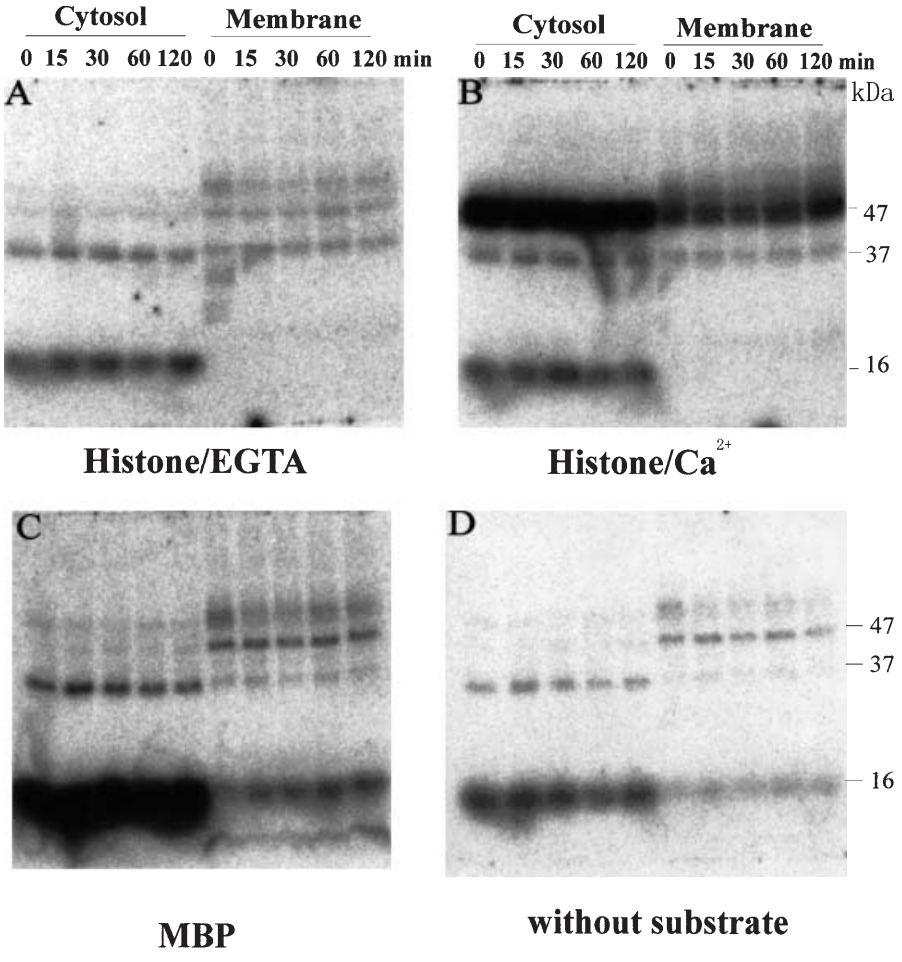


**Fig. 3A–D** CDPK activity induced by cold stress in cytosolic and membrane fractions of rice seedling stem tissues under light conditions in an early-response experiment. Stem tissues were collected after cold treatment for 0, 15, 30, 60 and 120 min. Cytosolic and membrane fractions were separated by 17% SDS-PAGE containing histone III-S (**A**, **B**). MBP (**C**) and without substrate (**D**)

stress signal transduction pathway (Sheen 1996), which is associated to changes in $Ca^{2+}$ (Knight et al. 1991, 1996) and protein phosphorylation (Anderberg and Walker-Simmons 1992).

## Comparison of the CDPK activities in aging stem segments and intact seedling stems

Differences in CDPK activity were checked among the 8-day-old rice seedlings and 5-week-old seedlings after a 5°C cold stress for 6 h. The 47-kDa CDPK activity in the cytosolic fraction remained stable between the cold treatment group and the control (25°C) (data not shown) but was induced in an increasing manner in the membrane fraction after cold stress relative to the negligible induction in the control. However, the intensity of this CDPK activity was higher in 5-week-old rice seedlings than in 8-day-old seedlings even though the induction patterns were quite similar (Fig. 4C). These data indicate that the CDPK activity induced by cold stress is not significantly different among seedlings of different ages, except that the intensity increases in older plants (5-week-old seedlings).

CDPK activity was compared between cold-stressed stem segments and stems from cold-stressed intact seedlings. As the CDPK activity induced in an increasing manner is present in the membrane fraction after a 6-h cold treatment, only membrane fractions were employed for this experiment. The 47-kDa CDPK activity was induced in an increasing manner by cold stress and reached a maximum at 6 h in the intact rice seedling stems; the change in stem segments was very similar to that of the intact seedlings, but the maximum level was reached at 3 h (Fig. 4A, B). These results show that there are no significant differences between intact seedlings and stem segments with respects to CDPK activity following a cold treatment. In addition, they provide us with a convenient and readily usable experimental material, stem segments, for use in inhibitor studies.

## Characterization of CDPK activity and its inducibility by various factors

Following determination of the 47-kDa protein kinase in stem tissues as being $Ca^{2+}$-dependent, some chemicals known to enhance or block CDPK activity and biotic or abiotic factors were employed in order to study their effects with an aim to further characterize this CDPK. $Ca^{2+}$ ionophore A23187, a $Ca^{2+}$ channel enhancer, can increase the $Ca^{2+}$ flowing into the cell or cytoplasm, with

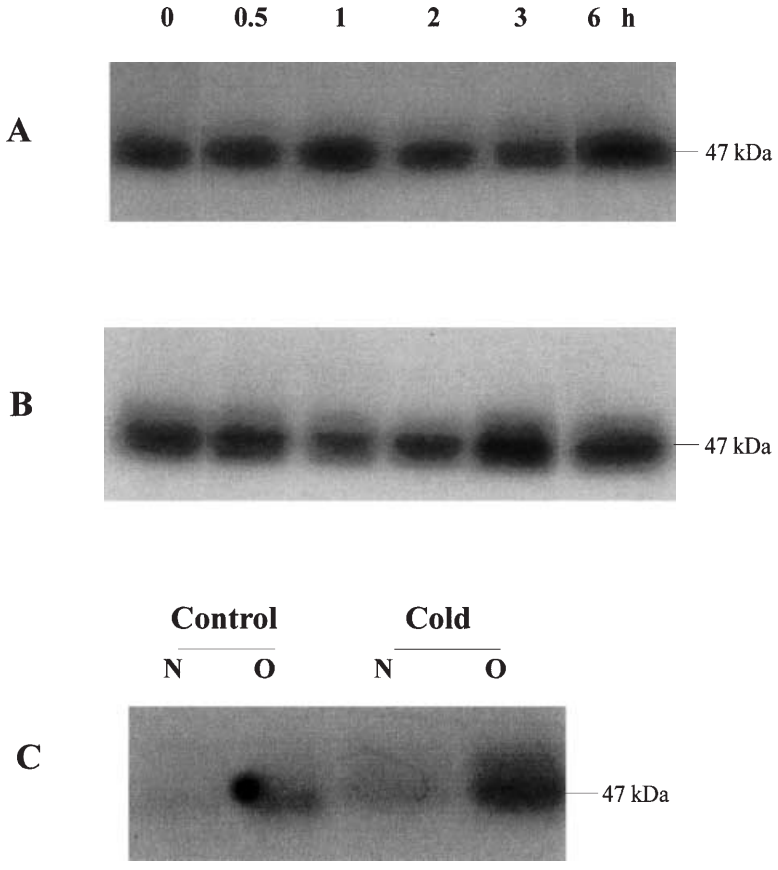


**Fig. 4A–C** Comparison of CDPK activity in rice seedling stems of intact plants and stem segments. Intact seedling stems (**A**) and stem segments (**B**) were collected after cold treatment at 5°C for 0.5, 1, 2, 3 and 6 h, and membrane fractions were separated by 17% SDS-PAGE containing histone III-S. **C** Eight-day-old seedling (*N*) and 5-week-old seedling (*O*) stem segments were cold-treated at 5°C for 6 h (including an initial 3 h under dark conditions).

the effect of increased CDPK activity. Our results show that A23187 improved the CDPK activity in the presence of $Ca^{2+}$ (Fig. 5) and did so in a dose-dependent manner. $LaCl_3$, a $Ca^{2+}$ channel blocker, and EGTA, a $Ca^{2+}$ chelator, were able to reduce DCPK activity a considerably, again indicating that the 47-kDa protein kinase is dependent on $Ca^{2+}$. The general protein kinase inhibitor, staurosporine, exhibited different inhibition kinetics, with a slight reduction and a complete inhibition of the 47-kDa CDPK activity in an in vivo (data not shown) and in vitro experiment, respectively. Protein kinase C (PKC) inhibitor, H7, partially blocked this CDPK activity in a dose-dependent manner, with high concentrations showing stronger inhibitory effects against this CDPK. The 47-kDa CDPK activity was decreased by the calmodulin antagonist, W7, in a manner similar to that of H7, which indicates that this CDPK has a calmodulin-like domain (Stone and Walker 1995). Okadaic acid, a protein phosphatase inhibitor, increased the 47-kDa activity – as it inhibits dephosphorylation – and caused a net increase in protein phosphorylation (Haystead et al. 1989).

In the immunobloting analysis, no cross reaction with anti-PKC antibody (Seikagaku Kogyo Co., Tokyo) was found in the stem tissues (data not shown). CDPK has been shown to cross-react with a mammalian protein kinase C (PKC) antibody an immunobloting analysis of the membrane of rice seeds (Abo-el-Saad and Wu 1995) and coleoptiles (Morello et al. 1993), but it seemed that different CDPKs existed in varying tissues and this 47 kDa CDPK may be specific than others.

We also investigated the effects of salt stress (NaCl), drought stress and phytohormone ABA (Fig. 5) on CDPKs, with the results showing an increased CDPK activity induced by these stresses, in which the NaCl and ABA treatments exhibited a stronger induction than drought stress. A 55-kDa rice membrane CDPK was reported to be induced by gibberellin, whereas no mammalian PKC or other plant CDPKs are known to be induced by any plant hormones (Abo-el-Saad and Wu 1995). However, it was recently reported that $Ca^{2+}$-dependent protein kinases activate ABA responses by constitutive protein phosphatase 2C activity (Sheen 1998). Also, our results primarily indicate that the 47-kDa CDPK in rice seedlings is affected by ABA in a multi-response regulating way. It is possible that this response, in which 47-kDa CDPK activity was induced in an increasing manner by salinity and drought, is due to the increased concentration in cystolic free $Ca^{2+}$ (Knight et al. 1997).

## Cold-induced CDPK activity is dependent of rice varieties

CDPK activity was further examined in different rice varieties, including cold-tolerant rice varieties (Kitaibuki, Yukihikari, Akita 39, Akihikari, Akitairi, Takaneminori,

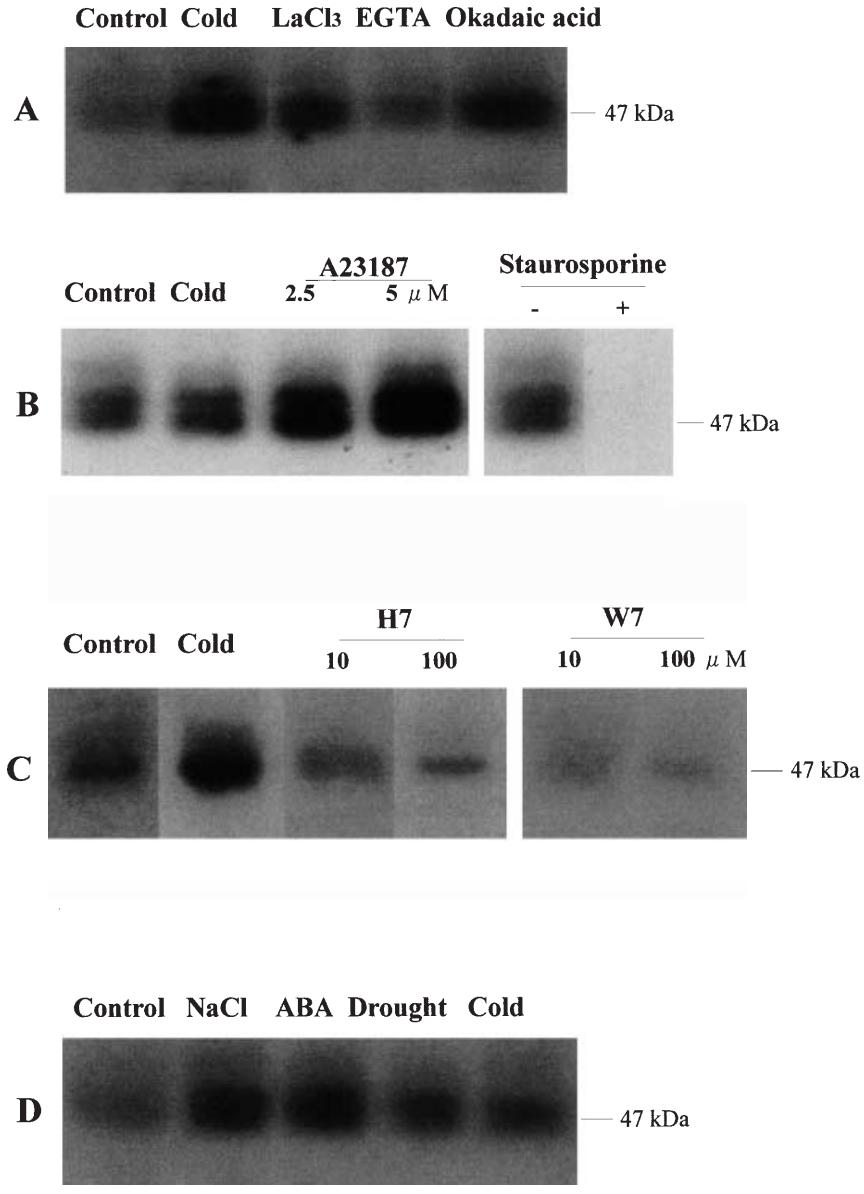


**Fig. 5A–D** The effects of chemicals and factors on CDPK activity in stem segments. The stem segments were floated on Milli Q water in plastic petri dishes and incubated at 5°C (cold treatment) for 6 h with the following additions. **A** 5 m*M* $LaCl_3$, 5 m*M* EGTA and 25 n*M* okadaic acid were used to characterize CDPK. **B** A23187/2.5 or 5, 100 μ*M* A23187 plus 2.5 or 5 m*M* $CaCl_2$ and 1 μ*M* staurosporine were used. **C** H7–10, H7–100, W7–10 and W7–100; the values indicate the working concentration in micromoles of inhibitors used. **D** 250 m*M* NaCl, 50 μ*M* ABA and drought (until stem segments lost 50% fresh weight) were also used to examine CDPK responses

Hayakogane and Hayamasari), cold-sensitive rice varieties (IR 36, ARROZ) and intermediate varieties (Nipponbare, Sensyo, Motsuhomare, Yamahoushi, Reimei, Sasanishiki, Hamaasahi and Akitakomachi), under cold stress, although the latter 8 varieties belong to the cold-tolerant japonica type, their cold resistance is slight lower than the former group (Yoshida and Kato 1994). Rice var. Nipponbare, which was used as the experiment material for this study, showed a CDPK activity induced by cold intermediate between Kitaibuki and IR 36.The other 15 rice varieties tested showed similar results; where CDPK activity was induced in an increasing manner after cold stress for 6 h in the stem membrane fractions, the activity induced in the cold-tolerant group was stronger than that induced in the cold-sensitive ones (Fig. 6). The activity of the 47-kDa CDPK was stable in cytosolic fractions among the 18 varieties (data not shown). However, the induction of CDPK activity in the stem membrane fractions was found to be dependent upon the rice variety (Fig. 6), with cold-tolerant rice varieties having a stronger response upon a 5°C cold stress treatment. One of the reasons for this is that the $Ca^{2+}$ influx increases during the cold acclimation process (Knight et al. 1996).

It is known that protein synthesis and mRNA levels are altered when plants are exposed to low temperatures (Thomashow 1990; Koga-ban et al. 1991). We previously reported that cold-sensitive rice varieties show a similarity in their leaf protein phosphorylation patterns in contrast to the cold-tolerant varieties (Komatsu and Kato 1997) and that a 35-kDa protein is more phosphorylated in cold-tolerant rice varieties (Komatsu et al. 1999). Protein phosphorylation and CDPK may be important in cold acclimation in rice varieties.

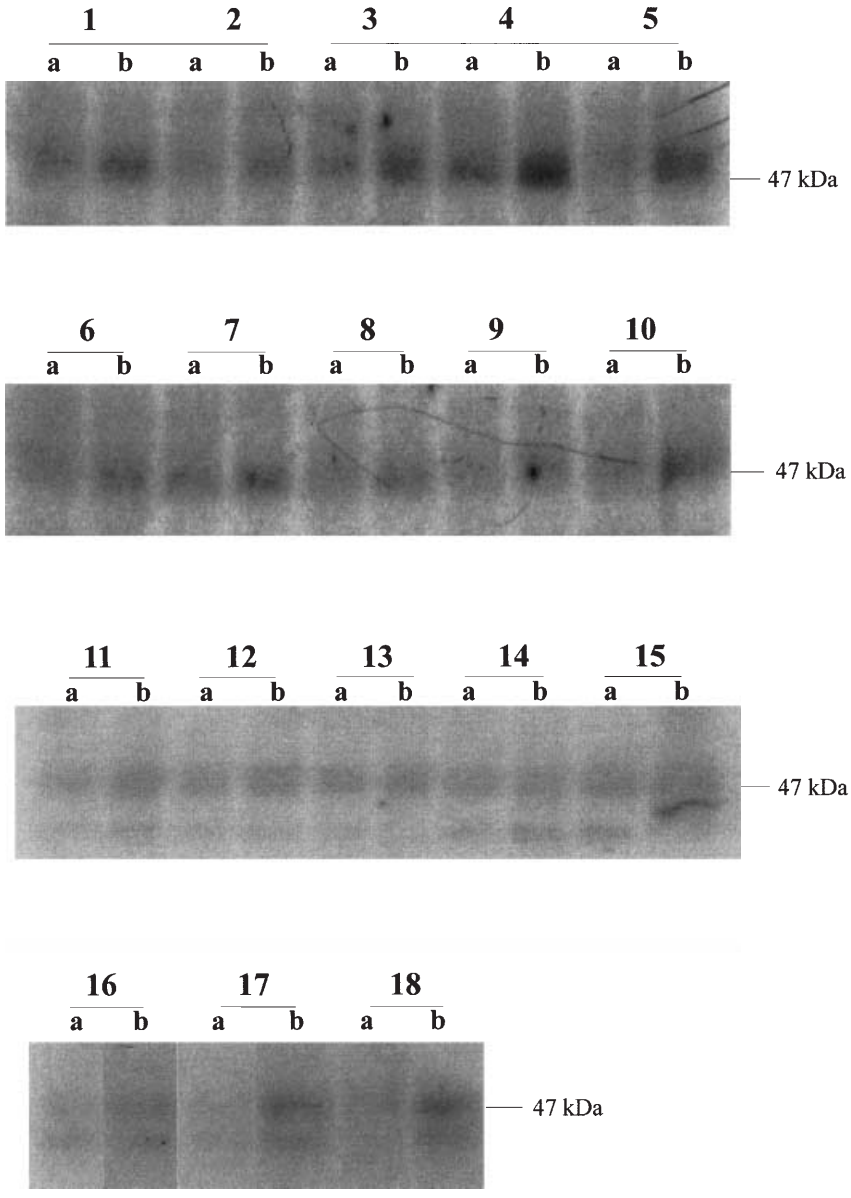


**Fig. 6** CDPK activity induced by cold stress in the membrane fraction of rice seedling stem tissues of different varieties. After cold treatment at 5°C (**b**) for 6 h, membrane fractions were collected, and in-gel phosphorylation assays were carried out on rice varieties Yukihikari (*1*), Sensyo (*2*), Akita 39 (*3*), Akihikari (*4*), Akitairi (*5*), Reimei (*6*), Takaneminori (*7*), Hayakogane (*8*), Akitakomachi (*9*), Sasanishiki (*10*), Hayamasari (*11*), Yamahoushi (*12*), Motsuhomare (*13*), Hamaasahi (*14*), ARROZ (*15*), IR 36 (*16*), Nipponbare (*17*) and Kitaibuki (*18*). *a* Control (25°C treatment)

In summary, these results show that the rice seedling can respond to cold stress and induce a 47-kDa CDPK in an increasing manner in stem tissues, and that the induction pattern of this CDPK is not significantly different between 8-day-old and 5-week-old seedlings although its response to a 5°C stress treatment was stronger among cold-tolerant rice varieties. This CDPK increased continuously in the stem tissue membrane fraction during the 12-h-long period following the cold stress. However, as this CDPK activity could also be induced by salt, drought and the phytohormone ABA, it seems that this CDPK is regulated not only under cold conditions but also by other stress factors.

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