

Characterization of a Ca^{2+} -Dependent Protein Kinase from Rice Root: Differential Response to Cold and Regulation by Absciscic Acid

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The Ca^{2+} -dependent protein kinases (CDPKs) and abscisic acid (ABA) are known to be involved in low-temperature stress response. The focus of this study was to characterize the 45 kDa protein kinase identified in the crude extract of rice (*Oryza sativa* L.) seedling roots in response to cold (5 °C) stress. The activity of the 45 kDa protein kinase decreased at low temperature as evident by an in-gel kinase assay using histone III-S as a substrate. Also, the Ca^{2+} -dependent activity of this protein kinase was suppressed by cold in the membrane fractions of the root. A general protein kinase inhibitor and Ca^{2+} chelator inhibited the activity of the 45 kDa protein kinase, suggesting that it was a plant CDPK. The 45 kDa CDPK identified was found to be independent of photosynthetic tissues such as the leaf and leaf sheath of rice seedlings, supporting a direct sensing mechanism in the roots of rice seedlings to cold stress. The suppressed activity of the 45 kDa CDPK was reverted by supplementing with 5 μM ABA under cold stress. The 45 kDa CDPK activity was stronger in the cold-tolerant variety of the 4 types tested than it was in the cold-sensitive one. These results suggest the involvement of endogenous ABA in regulating the activity of the 45 kDa CDPK in response to cold stress.

Key words *Oryza sativa*; root; cold stress; Ca^{2+} -dependent protein kinase; abscisic acid

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, and high or low temperature, which have profound effects on their growth and development. Rice seedlings can respond to cold treatment with major changes in gene expression.¹⁾ It is generally recognized that patterns of protein synthesis and mRNA level change when plants are exposed to cold.²⁾ Various stress conditions have been shown to influence protein phosphorylation.^{3,4)} During the early stages of cold acclimation, phosphorylation of cellular proteins and activation of protein kinases have been detected.⁵⁾ In our earlier study, the 60 kDa protein was independently phosphorylated in cold-tolerant Japonica varieties in cold treatment, but this phosphorylation is induced in cold-sensitive Indica varieties.⁶⁾ Recently, it has been reported that rice seedlings respond to cold stress of 5 °C by definite changes in protein phosphorylation.⁷⁾ This suggests that subjecting rice seedlings to low temperatures results in a variety of biochemical changes at the cellular level.

Low temperature treatment has been shown to induce an increase in cytosolic Ca^{2+} levels,^{8,9)} and Ca^{2+} could play an essential role in the cold-acclimation process.^{10,11)} Ca^{2+} plays important roles in numerous physiological processes and the presence of Ca^{2+} -regulated protein kinases will have wider implications in cellular signal transduction.^{12,13)} Ca^{2+} -dependent protein kinases (CDPKs) play a key role in stress and Ca^{2+} -mediated signal transduction. As a specific group of kinases CDPKs require only micromolar concentrations of Ca^{2+} for their activity, do not require calmodulin or phospholipids in plants and have been identified as Ca^{2+} -dependent and calmodulin-independent serine/threonine kinases.^{14,15)}

The plant CDPKs have been shown to phosphorylate a number of exogenous substrates, including histone III-S.¹⁶⁾ CDPKs exist as multiple isoforms¹⁷⁾ and isolation of cDNA further revealed that these enzymes are encoded by a multigene family.^{18,19)} As for their localization, CDPKs are either membrane-associated^{20,21)} or cytosolic in nature.^{22,23)} A 32 kDa CDPK was detected during regeneration of rice cultured suspension cells by an in-gel kinase assay.²⁴⁾ Also a 45 kDa Ca^{2+} - and phospholipid-dependent protein kinases in rice embryos and leaves were identified in previous studies.²⁵⁾ A 45 kDa CDPK, which can phosphorylate histone III-S in gel, was found in rice leaf under darkness in the presence of Ca^{2+} and phosphatidylserine²⁶⁾; their functions, however, remain to be clarified. Rice seedlings under cold stress induce a 47 kDa CDPK in an increasing manner in leaf sheath tissues.²⁷⁾ However, the function of CDPK is not clear in rice roots under cold stress.

It is known that phytohormone abscisic acid (ABA) has a wide variety of physiological, biochemical and molecular biological effects in plants. ABA levels increase in response to salt, cold, and high-temperature stress, each of which is known to be caused by a deficiency of water.²⁸⁾ It has also been shown that exogenous applications of ABA have the ability to harden plants against frost damage.²⁹⁾ In rice embryos, the phosphorylation of a 45 kDa protein kinase was increased by gibberellin and decreased by ABA.²⁵⁾ However, at the present time, the direct role for ABA in these processes remains unknown.

CDPKs play important roles in numerous physiological processes including the cold-stress response. The ABA is also known to be involved in the cold-stress response. The focus of this study is on the behavior of CDPK in relation to cold and ABA. In this study, the presence of phosphorylation activity in rice root under cold stress was investigated with the specific aim of identifying CDPKs activity. The influence of ABA on the CDPK activity was also examined in rice

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seedling root under cold-stress condition, suggesting the involvement of endogenous ABA in regulating the activity of the 45 kDa CDPK in response to cold stress.

MATERIALS AND METHODS

Plant Materials Rice (*Oryza sativa* L.) cv. Nipponbare, Kitaibuki, IR36 and Sasanishiki were used in this experiment. Seedlings were grown for 2 weeks in a growth chamber after germination under fluorescent light ($600 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, 12 h light and dark regime) at 25°C and 75% relative humidity. Two-week-old rice root segments were cut from seedlings, floated on pure water in Petri dishes, and subjected to cold stress in a 5°C cold chamber in the dark. The Nipponbare cultivar was used as the main material for this study. Root segments were homogenized after various treatments and analyzed as described below. Experiments were repeated three times.

Chemicals Ethylene glycol-bis (β -aminoethyl ether)- N,N,N',N' -tetraacetic acid (EGTA) and ABA were purchased from Wako Pure Chemical Industries (Osaka, Japan). Histone III-S was from Sigma (St Louis, MO, U.S.A.) and staurosporine was from Seikagaku Kogyo (Tokyo, Japan).

Preparation of Protein Extract A portion (250 mg) of the rice root was removed and homogenized with 1 ml extraction buffer containing 50 mM Tris-HCl (pH 7.4) 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1 mM EDTA, $5 \mu\text{M}$ sodium vanadate and 1 mM phenylmethylsulphonyl fluoride (PMSF). The homogenates were centrifuged at 15000 rpm for 10 min in an RA-50JS rotor (Kubota, Tokyo, Japan). The supernatant was used as the protein extract.

Preparation of Cytosolic and Membrane Protein Fractions A portion (250 mg) of the rice root was homogenized immediately with 500 μl of a homogenization buffer containing 20 mM Tris-HCl (pH 7.5) 0.25 M sucrose, 10 mM EGTA, 1 mM dithiothreitol (DTT) and 1 mM PMSF.²³⁾ The homogenates were centrifuged at 3000 rpm for 5 min and the supernatants were centrifuged at 100000 rpm for 15 min in a TLA 100.2 rotor (Beckman, CA, U.S.A.). The cytosolic fraction was obtained by collection of the supernatant. The pellet was washed in 100 μl of a homogenization buffer by centrifugation at 100000 rpm for 15 min. The pellet was resuspended in 50 μl of a membrane-solubilizing buffer containing 1% Triton X-100, 20 mM Tris-HCl (pH 7.5) 1 mM EDTA and 50 mM 2-mercaptoethanol, and solubilized on ice for 30 min. After centrifugation at 100000 rpm for 8 min, the membrane fraction was obtained by collecting the supernatant.

In-Gel Kinase Assay Protein extracts were separated on 17% sodium dodecyl sulfate (SDS)-polyacrylamide gels embedded with 2 mg/ml histone III-S as the substrate for the protein kinase in the separating gel.²⁶⁾ After electrophoresis, SDS was removed by washing the gel for 1 h with a buffer containing 50 mM Tris-HCl (pH 8.0) and 20% 2-propanol, and then for 1 h with a 50 mM Tris-HCl (pH 8.0) solution containing 5 mM 2-mercaptoethanol (Buffer A). The separated proteins were denatured for 1 h in Buffer A containing 6 M guanidine-HCl, and then allowed to renature by Buffer A containing 0.04% (w/v) Tween-40 at 4°C for 16 h. Proteins were reacted for 30 min at room temperature in the

reaction buffer containing 40 mM Tris-HCl (pH 8.0), 10 mM MgCl_2 , 2 mM DTT and 0.2 mM CaCl_2 with $50 \mu\text{M}$ [γ - ^{32}P] ATP (0.45 MBq, Amersham, Buckinghamshire, U.K.). 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, NY, U.S.A.).

Analysis of ABA Concentration After various treatments, roots were collected and crude ABA extracts were prepared in a homogenization buffer. Competitive ELISA was carried out using a Phytodetek ABA kit (Agdia, Elkhart, IN, U.S.A.) according to the manufacturer's instructions.

RESULTS AND DISCUSSION

CDPKs are a specific group of kinases that require only a micromolar concentration of Ca^{2+} for their activity and play an important role in numerous physiological processes, including the cold-stress response.²⁷⁾ The ABA is also known to be involved in low-temperature stress response.²⁸⁾ In this study, the presence of phosphorylation activity of CDPK in rice root after exposure to cold was investigated. The influence of ABA on the CDPK activity was also examined in rice root under cold-stress condition.

Roots were collected from rice 2 weeks after germination. Root segments were treated by 5°C cold and the crude proteins were extracted from treated roots. Protein extracts were separated by SDS-polyacrylamide gel containing histone III-S as a substrate in the separating gel, and an in-gel kinase assay was carried out in the reaction buffer containing 0.2 mM CaCl_2 . Protein kinase activity was detected by autoradiography. Forty-five-kDa and 17 kDa kinase activity was found in the presence of Ca^{2+} . Five-degree cold stress decreased the activity of a 45 kDa protein kinase in the crude extract of rice seedling roots within 2 h (Fig. 1A). On the other hand, cold stress increased the phosphorylating activity of a 17 kDa kinase (Fig. 1A). The 45 kDa protein kinase activity was detected in the membrane fractions but not in the

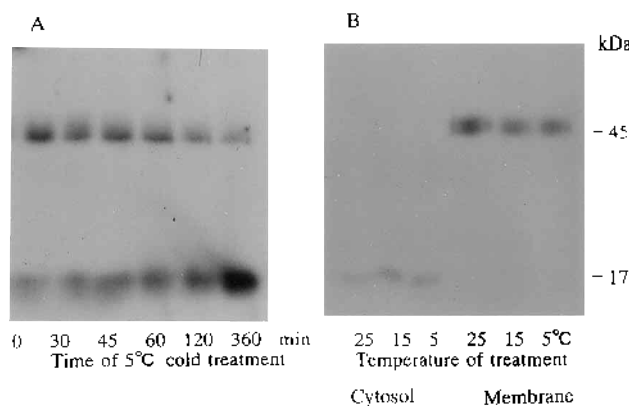


Fig. 1. Changes in Protein Kinase Activities during Cold Treatment in Root

Roots were collected at 14 d after germination. A) Root segments were treated for 30, 45, 60, 120, 360 min at 5°C . Total proteins were extracted from cold treated roots. B) Root segments were treated for 3 h at 5°C and the treatment at 25°C was used as control. Proteins were separated into cytosolic and membrane fractions. Proteins were reacted in the buffer containing 0.2 mM CaCl_2 using histone III-S as the substrate for in-gel kinase assay. Activities were detected by autoradiography. The positions of kinase activity were marked as 17 and 45 kDa. This is a typical figure of three experiments.

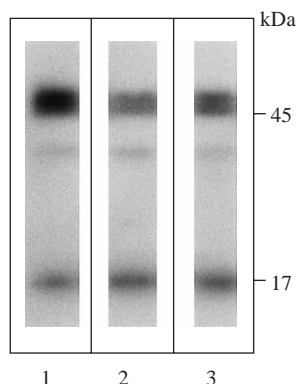


Fig. 2. The Effects of Chemicals on Protein Kinase Activity in Root

Roots were collected at 14 d after germination. Proteins were extracted and separated on SDS-polyacrylamide gel containing histone III-S as a substrate. One micromolar staurosporine (2) and 5 mM EGTA (3) were added to each reaction mixture. The control contained 0.2 mM CaCl_2 (1). Activities were detected by autoradiography. The positions of kinase activity are indicated as 17 and 45 kDa. This is a typical figure of three experiments.

cytosol fractions (Fig. 1B). Cold treatment decreased the phosphorylation activities of the 45 kDa protein kinase in the membrane fractions, and the 45 kDa protein kinase activity was most extensively decreased at 5 °C, compared with the phosphorylation at 25 °C or 15 °C (Fig. 1B). It is quite possible that numerous Ca^{2+} -dependent protein kinases exist in a plant as they are encoded by multi-gene families and show diversity in responsiveness to Ca^{2+} levels, expression patterns, subcellular localization, and physiological functions.^{14,30)}

To characterize the 45 kDa protein kinase identified in the present study, proteins extracted from roots were separated on SDS-polyacrylamide gel containing histone III-S as a substrate. One μM staurosporine and 5 mM EGTA were added to each reaction mixture. A Ca^{2+} chelator, EGTA, was able to reduce 45 kDa protein kinase activity (Fig. 2, lane 2), indicating that 45 kDa protein kinase has a Ca^{2+} -dependent activity. The general protein kinase inhibitor, staurosporine, inhibited 45 kDa protein kinase activity (Fig. 2, lane 3). These results suggest that the 45 kDa protein kinase is a plant CDPK. From the above results, it can be concluded that the 45 kDa kinase decreased by cold stress is a CDPK in rice root. The 47 kDa protein kinase as observed in the upper band of the 45 kDa protein kinase also has a Ca^{2+} -dependent activity, and the 47 kDa protein kinase activity was also decreased by cold stress. Since protein kinase inhibitors had no obvious influence on the 17 kDa kinase activity, 17 kDa kinase cannot be considered as a protein kinase. Its molecular mass and nature detected by the in-gel kinase assay were the same as previously reported and it was found to be nucleoside diphosphate kinase (NDP kinase) using an anti-NDP kinase antibody.³¹⁾ NDP kinases from various origins are auto-phosphorylated at the histidine residue, and the phosphoric group can be transferred to histone III-S.³²⁾

The change of the 45 kDa protein kinase activity in the root in response to cold stress was comparable between 2 weeks and 1 week after germination (Fig. 3A). The cold-stress response of the 45 kDa CDPK was independent of the photosynthesis of above-ground parts, which are leaf and leaf sheath of rice seedlings (Fig. 3B). This indicates a direct-sensing mechanism in the roots of rice seedlings to cold

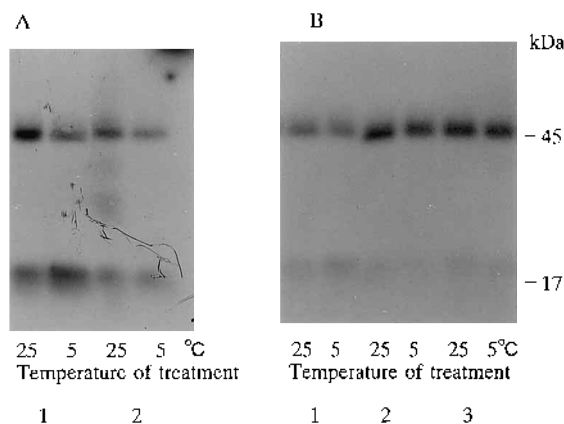


Fig. 3. Changes in CDPK Activities Affected by Cold Treatment in Root

A) Intact seedling roots were collected at 7 (2) or 14 (1) d after germination and treated for 3 h at 5 or 25 °C as control. B) Intact seedling roots (1), roots from seedling without above ground parts (2) and root segments (3) were treated at 5 °C for 3 h. CDPK activities were detected by autoradiography using histone III-S as a substrate for in-gel kinase assay. The position of kinase activity is indicated as 45 kDa. This is a typical figure of three experiments.

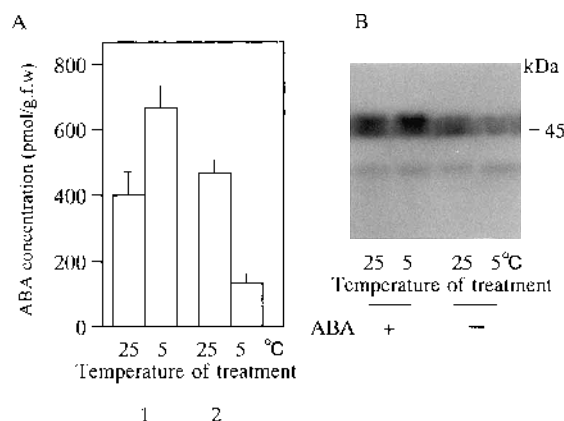


Fig. 4. Changes of Endogenous ABA Concentration and Effect of Exogenous ABA on CDPK Activity in Root under Cold Treatment

A) After 5 °C cold treatment for 24 h, roots from intact seedling were collected (1) and root segments were collected first and then treated in 5 °C cold for 24 h (2), and competitive ELISA was performed. Competitive ELISA was carried out using a ABA immunoassay detection kit (Agdia). B) Five micromolar ABA was added to water and combined treatment at 5 °C for 3 h was carried out. CDPK activity was detected by autoradiography after in-gel kinase assay. This is a typical figure of three experiments.

stress. Low temperature is an important factor affecting plant growth and development, that not only induces cold acclimation but also results in some extensive changes involved in the signal transduction pathway mediated through Ca^{2+} and protein kinase.

The focus of this study is on the behavior of this 45 kDa CDPK in relation to cold and ABA. ABA concentration increases in plant tissues under different stress conditions such as dehydration, high concentration of salts, and low temperature.²⁹⁾ In this study, the endogenous ABA concentration increased in intact root by cold stress (Fig. 4A-1). However, when 2-week-old rice root segments were cut from seedlings and floated on pure water in Petri dishes, the ABA concentration decreased in root segments by cold stress (Fig. 4A-2). In order to investigate the relationship of cold and ABA regulation on CDPK activity in roots, an in-gel kinase assay was carried out. Rice root segments were treated with cold stress in the presence of 5 μM ABA. The 45 kDa CDPK activity was found to be suppressed by cold in the membrane fraction

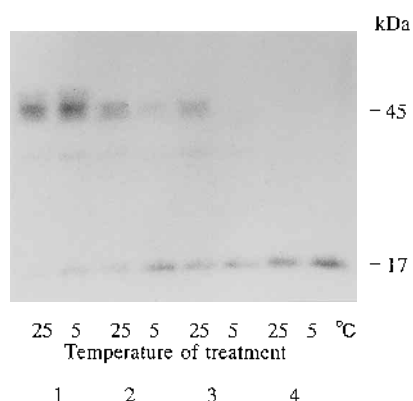


Fig. 5. Changes of CDPK Activity in the Membrane Fraction of Roots from Different Varieties under Cold Stress Condition

After cold treatment at 5°C for 3 h, membrane fractions were prepared from roots of different rice varieties, and in-gel kinase assay was carried out. Kitaibuki (1), Nipponbare (2), Sasanishiki (3) and IR 36 (4) were used in this study. CDPK activity was detected by autoradiography. This is a typical figure of three experiments.

of the root, but treatment with additional ABA recovered the suppression of the 45 kDa CDPK activity under cold-stress conditions (Fig. 4B). It has been reported earlier that exogenous applications of ABA have the ability to harden plants against frost damage.²⁹ These results suggest the involvement of the 45 kDa CDPK activity regulated by ABA in the cold-stress response in rice root. In rice embryos, the phosphorylation of a 45 kDa protein kinase was increased by gibberellin and decreased by ABA.²⁵ This result shows that there are several different pathways for signal transduction catalyzed by CDPK and ABA between the embryos and roots.

CDPK activity under cold stress was further examined in different rice varieties, including a cold-tolerant rice variety (Kitaibuki), a cold-sensitive rice variety (IR 36) and intermediate varieties (Nipponbare and Sasanishiki).²⁷ After cold treatment at 5°C for 3 h, the membrane fractions were prepared from roots of different rice varieties, and an in-gel kinase assay was carried out. Among the 4 rice varieties tested, the basic level of 45 kDa CDPK activity was the strongest in the cold-tolerant variety, Kitaibuki, and its activity was increased by cold stress, while the activity of this CDPK was greatly decreased in the intermediate varieties Nipponbare and Sasanishiki. In the cold-sensitive variety IR36, the activity of the 45 kDa CDPK was not detectable (Fig. 5). We have previously reported that the 60 kDa protein is irrespectively phosphorylated in cold-tolerant Japonica varieties in cold treatment, but this phosphorylation is induced in cold-sensitive Indica varieties,⁶ and that a 35 kDa protein is more phosphorylated in cold-tolerant rice varieties.⁷ In the leaf and leaf sheath of rice, 47 kDa CDPK activity was stronger in the cold-tolerant varieties than in the sensitive ones.²⁷ Protein phosphorylation and CDPK may be important in cold acclimation in rice varieties.

These results show that the rice root can respond to cold stress and that a membrane-located 45 kDa CDPK activity was decreased by cold, and recovered by exogenous ABA in

root segments. Its response to a cold-stress treatment was different among rice varieties with a different cold-tolerance test. It is indicated that a 45 kDa CDPK activity regulated by ABA is involved in the cold-stress response in rice.

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