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Partial Purification of OsCPK11 from Rice Seedlings and Its Biochemical Characterization

Jae-Hwa Shin[1] and Sung-Ha Kim[2]*

[1]*Gyeonggi Science High School, 135, Sooil-ro, Jangan-gu, Suwon-si, Gyeonggi-do 16297, Korea*
[2]*Department of Biology Education, Korea National University of Education, 250 Taesungtabyeon-ro, Gangnae-myeon, Heungdeok-gu, Cheongju-si, Chungbuk 28173, Korea*

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Calcium is one of the important secondary signaling molecules in plant cells. Calcium-dependent protein kinases (CDPK)－the sensor proteins of Ca^{2+} and phosphorylating enzymes－are the most abundant serine/threonine kinases in plant cells. They convert and transmit signals in response to various stimuli, resulting in specific responses in plants. In rice, 31 CDPK gene families have been identified, which are mainly involved in plant growth and development and are known to play roles in response to various stress conditions. However, little is known about the biochemical characteristics of CDPK proteins. In this study, OsCPK11－a CDPK in rice－was partially purified, and its biochemical characteristics were found. Partially purified OsCPK11 from rice seedlings was obtained by three-step column chromatography that involved anion exchange chromatography consisting of DEAE, hydrophobic interaction chromatography consisting of phenyl-Sepharose, and gel filtration chromatography consisting of Sephacryl-200HR. An *in vitro* kinase assay using partially purified OsCPK11 was also performed. This partially purified OsCPK11 had a molecular weight of 54 kDa and showed a strong hydrophobic interaction with the hydrophobic resin. *In vitro* kinase assay showed that the OsCPK11 also had Ca^{2+}-dependent autophosphorylation activity. The OsCPK11 phosphorylated histone III-S, and the optimum pH for its kinase activity was found to be 7.5~8.0. The native OsCPK11 shared several biochemical characteristics with recombinant OsCPK11 studied previously, and both had Ca^{2+}-dependent autophosphorylation activity and favored histone III-S as a substrate for kinase activity, which also had a Ca^{2+}-dependence.

Key words : Autophosphorylation, Ca^{2+}-mediated signaling, CDPK, column chromatography, OsCPK11, rice, transphosphorylation

Introduction

Among the molecules used in the signaling pathways of the plant cell, there is no molecule that represents the response to a variety of stimuli than the free Ca^{2+} in the cytoplasm [46]. Salinity/drought [28], hypo-osmotic stress [50], cold [27], heat shock [18], and NOD factors [15] have been found to cause the change of free [Ca^{2+}_{cyt}]. The ability of a single molecule to transmit information about various stimuli is due to Ca^{2+} signal magnitude, duration, frequency, location, and interactions with cellular structures and signaling pathways [36]. The unique signature of stimulus - specific Ca^{2+} signal is called the calcium signature [35]. Since Ca^{2+} is not metabolized in the cells, [Ca^{2+}_{cyt}] should be thoroughly controlled through various Ca^{2+}-binding proteins. For this, Ca^{2+}-binding proteins have the optimal structure to bind Ca^{2+}, and it plays a role in lowering [Ca^{2+}_{cyt}] or initiating a secondary signaling pathway [11].

In order for calcium to act as a secondary signaling molecule in the intracellular signal transduction, the dormant [Ca^{2+}_{cyt}] should be kept at a very low level. Conversely, given a particular stimulus, the [Ca^{2+}_{cyt}] is elevated at a rapid rate to initiate a specific response. It should also be consumed very quickly to go back to the idle state again for the next signal transfer [23]. Temporally increased recognition of free Ca^{2+} in the cytoplasm is associated with a primary Ca^{2+} sensor or a specific substrate on the signaling pathway [46].

Ca^{2+}-dependent protein kinases (CDPKs) are primary Ca^{2+} sensors that bind to high [Ca^{2+}_{cyt}] and are a unique class of kinases that transmit signals by transferring phosphoryl

***Corresponding author**
Tel : +82-43-230-3738, Fax : +82-43-232-7176
E-mail : plantedu@gmail.com

groups to cellular substrate proteins [23]. They are also the most abundant serine/threonine kinases in plants [48]. CDPKs are found in vascular plants, non-vascular plants, green algae and some protozoa [21]. CDPKs are encoded by the multi-gene family [52], and *Arabidopsis* (34) [24], rice (29)[6], and maize (35)[32] carry the CDPK genes. CDPKs also exist in a variety of cellular locations, indicating that they may be involved in a variety of signaling pathways [34]. Harmon *et al*. [19] suggested that CDPKs are involved in the potential regulation of gene expression, metabolism and signal transduction, migration of various ions and water through the membrane, and the dynamic movement of the cytoskeleton.

Indeed, as a Ca^{2+} sensor and a protein kinase, CDPKs regulate the diverse functions of plants, and the biological function of each CDPK isoform has been demonstrated in several plants. CDPKs are known as modulators of plant growth and developmental processes. Expression of pollen-specific CDPK genes in corn [16] and in *Arabidopsis* [38] are examples. CDPKs are also involved in hormone delivery and biotic and abiotic stress signaling [47]. In connection with the signaling of abscisic acid (ABA), several CDPKs in *Arabidopsis* play a central role in environmental stress responses, including drought, salinity, and cold [4]. CDPKs are also involved in the signal transduction pathways required to defend against infectious and herbal feeding [43]. They regulate various metabolic enzymes [49] and attenuate Ca^{2+} signal [14]. Although various functions performed by these cells have been revealed, but much still remains unknown.

Asano *et al*. [6] showed 29 CDPK genes in rice by a genome-wide analysis. Ray *et al*. [41] confirmed that 31 rice CDPK genes were identified by adding two new genes, and that each CDPK was involved in specific organ development and specific developmental stages. CDPK isoforms have been shown to be tissue-specific [6, 52]. Especially, OsCPK8 and OsCPK19 (OsCDPK2) were found in the panicle, while OsCPK10, OsCPK24 and OsCPK29 were mainly expressed in roots, and they were related to signal transmission in the panicle and root, respectively [52]. Each CDPK isoform has a different function in the cell, which can be broadly summarized as being involved in the growth and development of rice and in the signaling pathway of response to various stresses.

It is almost certain that rice CDPKs are involved in the growth and development. First, in the study using OsCPK2 and OsCPK11 [currently OsCPK7 based on Asano *et al*. [6] mRNA, it was confirmed that mRNA amount of *OsCPK2* in white light and anaerobic treatment was much lower than that of *OsCPK11* [8]. In addition, in a study by Frattini *et al.* [17] OsCDPK2 protein is expressed at a low level in the early stage of seed development, but later increased in amount and OsCDPK11 shows the opposite pattern. OsCDPK11 transcript and protein in leaves are not affected by light. While OsCDPK2 protein is almost absent upon exposure to light, it increases rapidly at night. It suggested that two CDPK isoforms perform different functions in response to seed development and light. This suggests a similar conclusion in Morello *et al*. [37] where the stability of the OsCDPK2 protein is regulated by light and is also involved in the seed formation. In the study of the location of CDPKs in the cells, OsCPK2 was found to be associated with the membrane by the myristoylation and palmitoylation [34].

There are many studies that rice CDPKs are involved in various stress responses. Wan *et al*. [52] reported that the promoter region of *OsCPK1~OsCPK29* genes contained multiple stress-responsive *cis*-elements and that CDPK genes were extensively involved in the stress responses. OsCDPK7 belongs to stress-inducible CDPKs, and their transcripts increase by a low temperature and salt stress conditions [45]. Under the low temperature stress condition, the role of ABA in controlling the activity of 45 kDa CDPK in rice was determined. In addition to OsCDPK13, which was found to be stress-inducible [1], OsCPK6, OsCPK17, and OsCPK25 were also found to be very important in stress tolerance of rice [52]. OsCPK21 and OsCPK12 are positive regulators of the salt stress signaling pathway [5]. Like the CDPKs of other plant species, many of the unique biological functions of CDPKs in rice remain largely unknown.

The first study to purify and characterize plant CDPKs was performed by Harmon *et al.* [20] They used CDPKs from soybean to validate the key properties of CDPKs that require calcium for enzyme activity, but do not require calmodulin. This is due to structural features with a calmodulin-like domain that can bind directly to calcium at the C-terminal of CDPKs [21]. In rice, it was the first obtained by a partial purification of CDPK in leaves and its activity was confirmed to be calcium dependent [25]. The activity of CDPK purified from sandalwood [3], and beetroot cell membranes [30] depends on calcium precisely. In addition, CDPKs had a unique biochemical characteristics in autophophorylation

and transphosphorylation of specific substrates. In the kinase assay using CDPK1 of chickbean (*Cicer arietium*) expressed in *E. coli*, calcium-dependent autophophorylation and transphosphorylation were confirmed [13]. Cho [10] studied the biochemical characteristics of recombinant OsCPK11 expressed in *E. coli*. In this study, a biochemical characteristics of native OsCPK11 obtained from rice leaves was investigated. And the similarities and differences between native OsCPK11 and recombinant OsCPK11 will be discussed.

Materials and Methods

Materials

Rice (Nipponbare) seeds were sown on MS medium and grown for 2-3 weeks. At this time, a chamber having a constant temperature of 30℃, and a photoperiod of 16 hr light/8 hr dark was used. Leaves were harvested and used for the purification experiment. Most chemicals used in this study are from Sigma Aldrich (USA) as follows; 2-amino-2-(hydroxymethyl)-1,3-propanediol (Trizma base), sodium chloride, magnesium chloride, calcium chloride, ammonium sulfate, sodium phosphate monobasic, potassium phosphate monobasic, glycine, polyethylene glycol sorbitan monolaurate (Tween-20), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 2-(N-morpholino)ethanesulfonic acid hydrate (MES), adenosine 5′-triphosphate (ATP), sodium orthovanadate (Na_3VO_4), ethylene glycol-bis(2-aminoethylether)-N,N,N,N′-tetraacetic acid (EGTA), phenylmethanesulfonyl fluoride (PMSF), ethylenedinitrilo tetraacetic acid (EDTA), 1,4-dithiothreitol (DTT), histone Ⅲ-S, myelin basic protein and casein. Methanol is the product from Emsure. [γ^{32}-P] Adenosine 5′-triphosphate (6,000 Ci/mmol) was from Perkin Elmer (Boston, USA).

Purification of OsCPK11

The rice leaves grown for 2-3 weeks were harvested and their proteins were extracted. Rice leaves was measured and at least 1 ml of protein extraction buffer (50 mM Tris, 150 mM NaCl, 10 mM $MgCl_2$, 1 μM approtinin pH 7.5) per 100 mg of the fresh weight was added and ground it finely with a grinder. Crude extract was filtered through gauze and miracloth (Millipore, USA), then centrifuged at 11,000× g for 20 min at 4℃ using centrifuge (Model Avanti JXN-30, Beckman). The supernatant was collected and used for the purification.

Column chromatography

Chromatography was carried out using a biologic LP system (Bio-Rad, USA) in a 4℃ chamber. First, gel filtration chromatography was performed to remove low molecular substances including salts contained in the crude extract. At this time, 30 ml of crude extract was desalted at once using Bio-Gel P-6 desalting cartridge (Bio-Rad, USA, 50 ml) and 25 mM Tris buffer (pH 8.0). Desalted crude extract was collected and proceeded to the anion exchange chromatography. 5 ml of DEAE Sepharose (GE Healthcare, USA) was packed in an open column (1×10 cm, Bio-Rad, USA). Start Buffer was 25 mM Tris buffer (pH 8.0) and Binding Buffer was 25 mM Tris buffer (pH 8.0) containing 1 M NaCl. Equilibrium was performed for 200 min with Start Buffer. After 25 min, sample was loaded instead of the Start Buffer. In the gradient, the proteins attached to the resin were eluted by increasing the salt concentration from 0% to 100% by mixing the Start Buffer and the Binding Buffer for 80 min. And eluted proteins were collected in each 1 ml fractions. After elution of all the proteins attached to the resin with Binding Buffer for 25 min, re-equilibrium was performed with Start Buffer for 50 min. The flow rate was kept constant at 1 ml/min. Through buffer exchange with Start Buffer (50 mM sodium phosphate buffer containing 1.5 M ammonium sulfate, pH 7.5) for the next purification step using Centriprep 10K (Miliipore, Germany), a final sample was obtained to carry out the next step.

In order to perform hydrophobic interaction chromatography, 3 ml of Phenyl-Sepharose (GE Healthcare, USA) was packed in an open column (1×10 cm, Bio-Rad, USA). Start Buffer was 50 mM sodium phosphate buffer (pH 7.5) containing 1.5 M ammonium sulfate and the Binding Buffer was 50 mM sodium phosphate buffer (pH 7.5). Equilibrium was performed for 75 min with Start Buffer. After 15 min, sample was loaded instead of the Start Buffer. In the gradient, the proteins attached to the resin were eluted by decreasing the salt concentration from 100% to 0% by mixing the Start Buffer and the Binding Buffer for 60 min. And eluted proteins were collected in each 2 ml fractions. After elution of all the proteins attached to the resin with Binding Buffer for 15 min, re-equilibrium was performed with Start Buffer for 30 min. The flow rate was kept constant at 1 ml/min. As in the previous step, fractions which contained target proteins were combined and concentrated with Centriprep 10K (Miliipore, Germany).

As a final step, gel filtration chromatography using

Sephacryl-200HR (Sigma Aldrich, USA) was performed. Forty-two milliliter of Sephacryl-200HR was used to pack the open column (1×30 cm, Bio-Rad, USA) and PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) was used for the process, and the flow rate was maintained at 0.4 ml/min. After loading the sample on the packed resin, PBS buffer was flowed for 100 min. Finally, fractions containing target proteins were combined and concentrated using Centriprep 10K (Miliipore, Germany). In order to confirm the efficient the purification process, several confirmation steps were carried out. Total protein obtained from each purification step was measured. Samples were as follows: ⅰ) desalted crude extract, ⅱ) sample eluted from anion exchange chromatography, ⅲ) sample eluted from hydrophobic interaction chromatography and ⅳ) fractions from gel filtration chromatography. Total protein content was measured using the BCA Protein Assay kit (Sigma Aldrich, USA) [31].

SDS-PAGE

SDS-PAGE was performed using a stepwise sample to confirm protein pattern in each purification process. Protein separation was performed using SDS-PAGE mini kit (Major Science, South Korea). The sample was loaded into each well of 10% SDS-PAGE gel and protein separation was performed. Gel obtained after electrophoresis was stained with Coomassie Blue solution and protein bands were observed.

Western Blot

Fractions containing the target proteins in each purification step were confirmed by western blot using OsCPK11 antibody. Purified OsCPK11 antibody was prepared and used. Synthetic peptide was made based on known amino acid sequence of OsCPK11, and it was injected into the rabbits to induce antibody formation. Serum from the blood collection was purified and OsCPK11 antibody with high affinity was obtained. Western blotting was performed on all fractions obtained from the gradient section of DEAE chromatography and Phenyl-Sepharose chromatography, and the fractions centered on the peak of Sephacryl-200HR chromatography. After selecting the samples to be analyzed for each purification step, SDS-PAGE was performed. Gel was transferred to PVDF membrane (Immobilon-P, Millipore, Germany). Transfer from the gel to the PVDF membrane was carried out in a Transfer Buffer (25 mM Tris, 192 mM glycine, 20% methanol) for 90 min at 4℃ and 100V (Electro Blot System, Major Science, South Korea). Blocking was done at room temperature for 3 hr in Blocking Solution (5% skim milk in 0.1% PBST). It was treated with OsCPK11 antibody (1:2,000 dilution) in 2.5% skim milk, and the membrane was reacted at room temperature for 3 hr, and then the wash step was initiated. It was washed with 2.5% skim milk in 0.1% PBST twice for 15 min, and with 0.1% TBST for 15 min once. And reaction was done at room temperature for 1 hr in 2.5% skim milk containing secondary antibody (anti-rabbit IgG AP conjugate, Promega, USA; 1:5,000 dilution). After washing with 0.1% TBST twice for 15 min and once for 15 min with TBS, color development was performed in alkaline phosphate buffer (100 mM Tris, 150 mM NaCl, 1 mM $MgCl_2$, pH 9.0) containing NBT and BCIP (Promega, USA) to confirm the final band pattern.

Autophosphorylation assay

Autophosphorylation was performed *in vitro* using a partially purified OsCPK11 as described before. 4X Kinase Reaction Buffer (20 mM HEPES, 200 nM ATP, 1 mM Na_3VO_4, 2 mM DTT, 0.5 mM PMSF, 2 mM EDTA, pH 7.4) was prepared. 0 or 100 μM of $MgCl_2$, 0 to 1 mM $CaCl_2$, 1 mM of EGTA, 1 μg of OsCPK11 were added to each Reaction Buffer, and the final volume was adjusted to 12 μl with 20 mM HEPES (pH 7.4). Then, 10 UCi [γ^{32}-P]ATP diluted 10 fold with 20 mM HEPES was added and incubated for 0.5~1 hr at 30℃. Four microliter of 4X Sample Buffer was added and boiled for 5 min at 100℃. Sample was loaded into each well of 12% SDS-PAGE gel and protein separation was performed. Gel was dried using gel dryer (Bio-Rad, Model 583) for 30 min at 80℃, and exposed to BAS plate for 2~3 days at -80℃. The film was developed and examined. The intensity of raidioactivity was calculated by Image J software (National Institutes of Health, USA).

Transphosphorylation assay

In order to examine the transphosphorylation properties of partially purified OsCPK11, following experiments were conducted. First, three different substrates of histone Ⅲ-S, myelin basic protein and casein were used to find the best substrate for a partially purified OsCPK11 *in vitro*. One microgram of each substrate, 1 μM of $CaCl_2$, 100 μM of $MgCl_2$, 1 mM of EGTA and 1 μg of OsCPK11 were added to the 4X Kinase Reaction Buffer. After this, rest of experimental procedure was the same as one described in autophosphorylation experiment.

In order to find the optimal pH for the transphosphorylation activity of partially purified OsCPK11, 1 μM $CaCl_2$, 100 μM $MgCl_2$, 1 μg of OsCPK11 and histone Ⅲ-S were added to 4X Kinase Reaction Buffer of pH 6 to pH 9. A series of different 20 mM buffer solution from pH 6 to pH 9 was added to each to make a final volume to 12 μl. After this, rest of experimental procedure was the same as one described in autophosphorylation experiment.

Results

Partial purification of OsCPK11

From DEAE chromatography, the OsCPK11 was eluted in the 17.5%~55% NaCl gradient section (0.175 M to 0.55 M) as shown in Fig. 1A, which was confirmed by the western blot analysis. At this step, OsCPK11 was presumed to have a weak anionic amino acid on its surface. From Phenyl-Sepharose chromatography, OsCPK11 was eluted in the 27%~0% $(NH_4)_2SO_4$ gradient section (0.4 M to 0 M) as shown in Fig. 1B, which was confirmed by the western blot analysis. This indicated that OsCPK11 has a strong hydrophobic binding Phenyl-Sepharose resin. Most CDPKs were characterized by their ability to bind strongly to hydrophobic resins such as Phenyl-Sepharose [42]. CDPKs of soybean, maize and silver beet were known to have hydrophobic binding sites [7, 40] and potato protein kinase was eluted by 4 M urea [33], and the salt-resistant CDPKs of *Dunaliella tertiolecta* were eluted with 50% of ethylene glycol [54]. This was also confirmed by the refining process of this study. From Sephacryl-200HR chromatography, fractions # 27 and # 28 contained partially purified OsCPK11 (Fig. 1C) and this was confirmed by western blotting.

Several confirmation procedure was performed to determine if the purification level proceeded effectively during the purification steps. The total protein content of each sample during purification steps was measured and result was shown in Table 1. During purification steps, fractions containing the target protein was recovered and the next step was carried out. At each step, Western blot analysis was per-

Table 1. Total protein content during purification step

Purification step	Total protein (mg)
Crude extract	1,187.3
DEAE-Sepharose column chromatography	67.1
Phenyl-Sepharose column chromatography	9.6
Sephacryl-200HR column chromatography	0.2

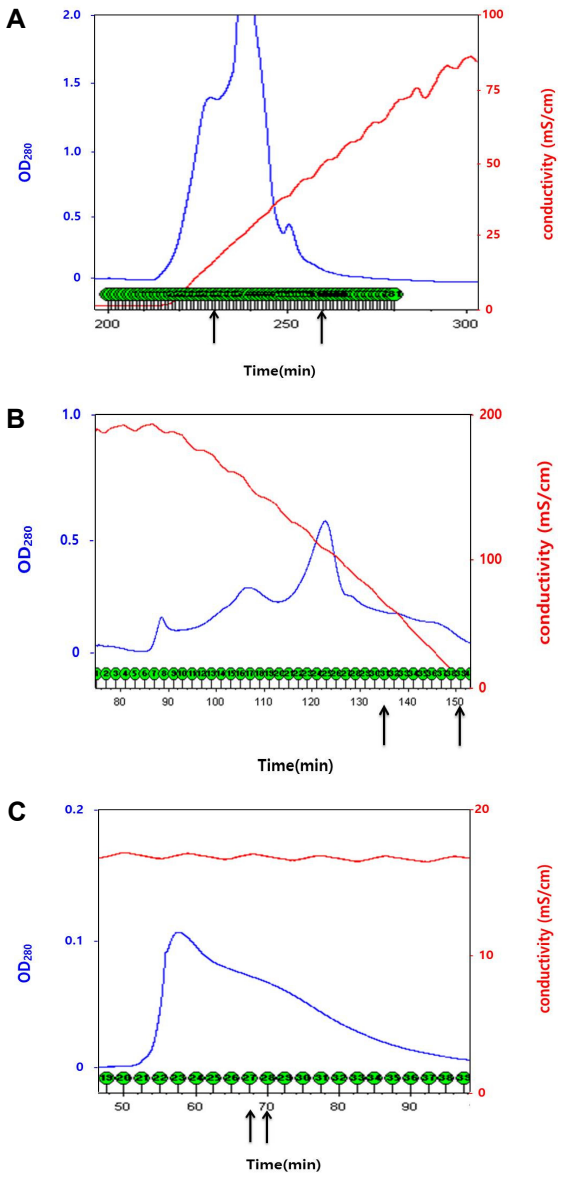

Fig. 1. A. Elution profile of DEAE column chromatography. It showed the gradient range between 200 and 280 min in the entire DEAE chromatography. 1 M of NaCl concentration was gradually increased from 0 to 100%, and the proteins bound to the resin were eluted. The interval indicated by (↑) mark was the interval over which OsCPK11 was eluted, which corresponds to 0.175 M to 0.55 M NaCl. B. Elution profile of Phenyl-Sepharose column chromatography. It showed the gradient range between 75 and 135 min in the entire Phenyl-Sepharose chromatography. Proteins bound to the resins were eluted by reducing the concentration of 1.5 M ammonium sulfate gradually from 100% to 0%. The interval indicated by (↑) mark was the interval over which OsCPK11 was eluted, which corresponds to 0.4 M to 0 M $(NH_4)_2SO_4$. C. Elution profile of Sephacryl-200HR column chromatography. The time in between two mark (↑) at the bottom indicates when OsCPK11 was eluted.

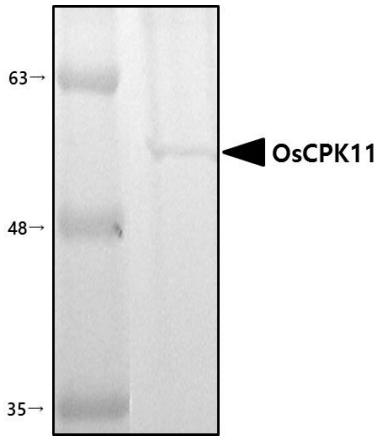

Fig. 2. Western blot analysis of the fractions from Sephacryl-200 HR column chromatography. Eluted samples from Sephacryl-200HR column chromatography were determined by Western blot analysis using OsCPK11 antibody. OsCPK11 was present in the fractions #27~#28. Its molecular weight was estimated to be 54 kDa.

formed to find OsCPK11, and results were shown in Fig. 2. The molecular weight of OsCPK11 was estimated to be 54 kDa, which was confirmed by western blotting as well as autoradiography (Fig. 3). This is similar to the study by Cheng *et al.,* [9] that CDPK is a monomer and has a molecular weight in the range of 40~90 kDa. The molecular weights of known CDPKs indicate 52 kDa from soybean [20, 40], 53 kDa from groundnut [12] and 45 kDa from barley [26].

In vitro kinase assay

Ca^{2+} and Mg^{2+} influence the activity of autophosphorylation of OsCPK11 (Fig. 3). In the absence of Mg^{2+}, autophosphorylation of OsCPK11 was activated, but when Ca^{2+} was absent, no activity of autophosphorylation was observed (Fig. 3), suggesting that Ca^{2+} is essential for the activation of autophosphorylation of partially purified OsCPK11. In the absence of Mg^{2+}, autophosphorylation activity was observed even in the presence of lower Ca^{2+}, while the activity of autophosphorylation was decreased when Ca^{2+} become higher (Fig. 3). Even when Ca^{2+} was at 1 mM, autophosphorylation was not activated at all. Conversely, when 100 μM Mg^{2+} was present, the autophosphorylation activity of partially purified OsCPK11 gradually increased as Ca^{2+} increased (Fig. 3). As a result, the strongest autophosphorylation activity was observed when 1 μM Ca^{2+} was present without Mg^{2+}, and the strongest autophosphorylation activity was observed when 1 mM Ca^{2+} was present with 100 μM Mg^{2+} (Fig.

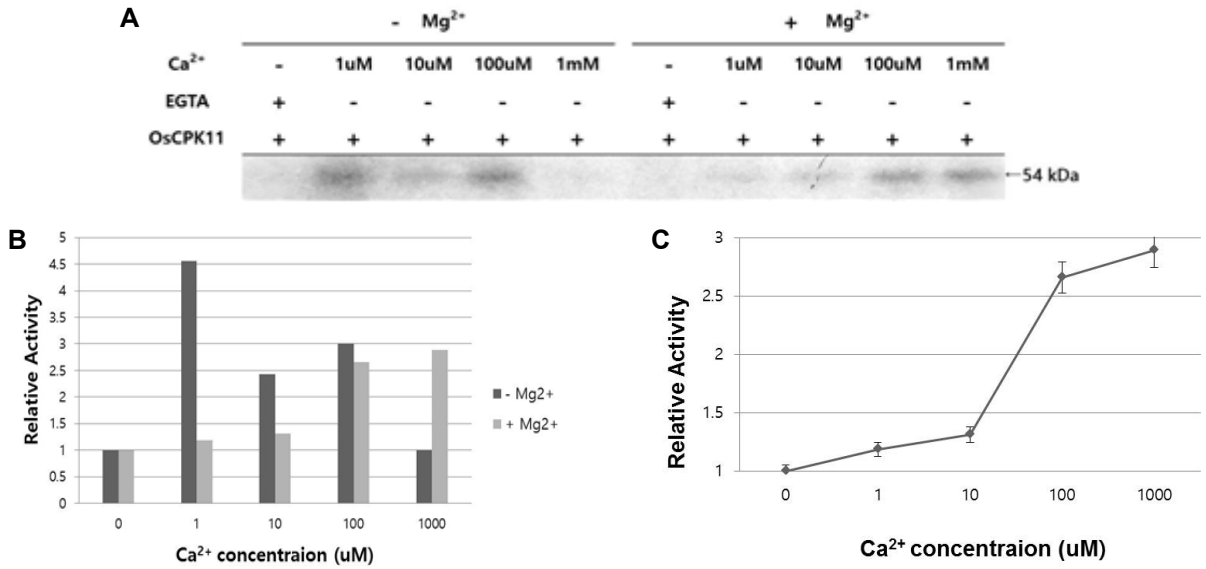

Fig. 3. Effects of Mg^{2+} and Ca^{2+} on the autophosphorylation activity of OsCPK11. A. Partially purified OsCPK11 showed a strong autophosphorylation activity at 1 μM Ca^{2+} in the absence of Mg^{2+}. In the presence of 100 μM Mg^{2+}, the autophosphorylation activity tended to increase as Ca^{2+} concentration increased, and the strongest autophosphorylation activity was observed at 1 mM Ca^{2+}. B. This showed the autophosphorylase activity of partially purified OsCPK11 with or without Mg^{2+} under different Ca^{2+} concentration. When Ca^{2+} was not present, no autophosphorylation activity was observed regardless of the presence or absence of Mg^{2+}. C. This showed the change of autophosphorylation activity of partially purified OsCPK11 as Ca^{2+} concentration increased in the presence of 100 μM Mg^{2+}. The higher the Ca^{2+} concentration, the greater the autophosphorylation activity of partially purified OsCPK11, and it had the highest activity at 1 mM Ca^{2+}.

3). Therefore, the partially purified OsCPK11 in this study has a Ca^{2+}-dependent autophosphorylation function. Numerous studies supported the autophosphorylation of serine/threonine residues in CDPKs is Ca^{2+}-dependent and independent of calmodulin [20, 22]. Conversely, winged bean CDPK has an autophosphorylation activity independent of calcium [44]. Little is known about the effects of autophosphorylation on CDPK function *in vitro*, and almost nothing is known about its physiological or mechanistic role *in vivo* [22]. Therefore, more extensive studies will be needed to examine the effect of Ca^{2+} on autophosphorylation of CDPKs.

Partially purified OsCPK11 in this study was tested with three different substrates: histone Ⅲ-S, MBP, and casein with or without 1 μM Ca^{2+}. Result showed that partially purified OsCPK11 strongly phosphorylated histone III-S, which was Ca^{2+}-dependent (Fig. 4). That is, kinase activity was stronger in the presence of Ca^{2+}. On the other hand, MBP and casein were almost not phosphorylated, as shown in Fig. 3. In addition, partially purified OsCPK11 and histone III-S were reacted in the presence of 1 mM Ca^{2+} and 100 μM of Mg^{2+}, and the pH of the reaction buffer varied from pH 6 to pH 9. Result indicated that partially purified OsCPK11 showed the strongest activity at a slightly alkaline pH 7.5~8.0 (Fig. 5). It was similar to the optimum pH range of the phosphorylation activity of CDPKs of other species [30]. As a special case, three types of soybean CDPKs have been known to have optimal pH 6-9 for the phosphorylation of syntide-2 [29].

Comparison of native OsCPK11 and recombinant OsCPK11

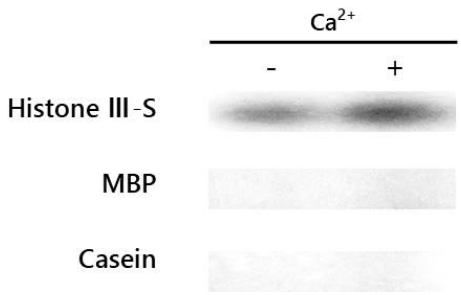

Fig. 4. Searching for the substrate for OsCPK11. One microgram of partially purified OsCPK11 was reacted with each 1 μg of three different substrates of histone Ⅲ-S, MBP and casein. Among them, histone Ⅲ-S seemed to be the best substrate for the partially purified OsCPK11 phosphorylase activity was the most strongly observed in histone III-S. It had a stronger kinase activity in the presence of 1 μM Ca^{2+}, while it did not phosphorylate either MBP or casein.

Biochemical characteristics of OsCPK11 identified in this study could be compared in some aspects with the characteristics of recombinant OsCPK11 identified studied by Cho [10]. Results were shown in Table 2.

Native OsCPK11 and recombinant OsCPK11 all had Ca^{2+}-dependent autophosphorylation activity. However, there are some differences. Native OsCPK11 showed the autophosphorylation activity at 1 μM Ca^{2+} in the absence of Mg^{2+}, whereas recombinant OsCPK11 did not show any autophosphorylation activity.

In the presence of Mg^{2+}, native OsCPK11 showed an increase in autophosphorylation activity as Ca^{2+} concentration increased. However, recombinant OsCPK11 showed autophosphorylation activity was almost constant when the Ca^{2+} concentration was increased. This difference needs to be studied further.

Both native OsCPK11 and recombinant OsCPK11 preferred histone Ⅲ-S as a substrate for the kinase activity *in vitro*, and that kinase activity was dependent on Ca^{2+}. This appears to be due to the structural similarity of the substrate binding sites and calcium binding sites of both proteins. Partially purified native OsCPK11 and recombinant OsCPK11 phosphorylated histone III-S, but further studies are needed to determine if OsCPK11 has a function associated with histone III-S in plant cells.

Both the native OsCPK11 and the recombinant OsCPK11 were similar in terms of optimal pH range of the kinase activity *in vitro*. There was a little bit of difference between native OsCPK11 in the range of pH 7.5~8 and the recombi-

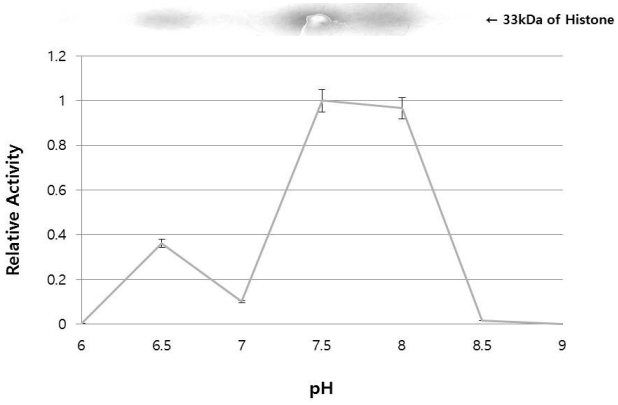

Fig. 5. Optimal pH for the transphosphorylation activity of OsCPK11. Partially purified OsCPK11 and histone III-S were reacted at the different pH from pH 6 to pH 9, respectively. Each buffer contained 1 mM Ca^{2+} and 100 μM Mg^{2+} as well. It showed the strongest activity at a slightly alkaline pH 7.5~8.0.

Table 2. Comparison of native OsCPK11 and recombinant OsCPK11

	Native OsCPK11	Recombinant OsCPK11
Autophosphorylation	+	+
Transphosphorylation-optimal substrate	Histone Ⅲ-S	Histone Ⅲ-S, MBP
Transphosphorylation-optimal pH	pH 7.5~8	pH 7~7.5

nant OsCPK11 in the range of pH 7~7.5. There are many studies that showed native and recombinant proteins are functionally almost identical [39], and further study will be needed on the detailed differences between them.

There were some limitations in purifying OsCPK11 and revealing its biochemical characteristics. OsCPK11 was found to be a very small amount in plant cells and it was difficult to purify with a higher purity. Due to the non-specific binding characteristics of the OsCPK11 antibody used in western blot, it was sometimes difficult to differentiate and separate OsCPK11 band.

To confirm a correct biological function of autophosphorylation and transphosphorylation of CDPKs, experiments should be needed *in vivo*. And it will provide a more extended knowledge of the role of OsCPK11 in signaling pathways in plant cells. This study will provide a basic knowledge in making such an extended study.

Discussion

In this study, OsCPK11, one of CDPKs from rice, was partially purified from rice seedlings. The partial purification steps included anion exchange chromatography, hydrophobic interaction chromatography and gel-filtration chromatography. OsCPK11 seems to have a weak anion on the surface and a strong hydrophobic bond with the hydrophobic resin (Fig. 1). It was found that molecular weight of the subunit was estimated to be 54 kDa. Its *in vitro* kinase assays also showed that it had a Ca^{2+}-dependent autophosphorylation activity and was influenced by Mg^{2+} (Fig. 3). Especially, it was found that autophosphorylation activity increases with increasing concentration of Ca^{2+} in the presence of 100 μM of Mg^{2+} (Fig. 3). In addition, partially purified OsCPK11 was found to phosphorylate histone III-S strongly (Fig. 4), and it was found that the optimum pH range was pH 7.5-8.0 (Fig. 5). The biochemical characteristics of this native OsCPK11 were compared with those of recombinant OsCPK11 [10]. Both OsCPK11 have Ca^{2+}-dependent autophosphorylation activity and histone Ⅲ-S is preferred as a substrate for the kinase activity, and the optimum pH is about 7.5(Fig. 3). These results should help understanding the biochemical characteristics of OsCPK11. If we extend the experiments *in vivo*, we will be able to find the physiological function of OsCPK11 in rice.

The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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초록 : 벼 유식물에서 OsCPK11의 부분 정제 및 생화학적 특성 규명

신재화[1] · 김성하[2*]
([1]경기과학고, [2]한국교원대 생물교육과)

식물에서 Ca^{2+}는 세포의 중요한 2차 신호 전달 분자 중 하나이다. Ca^{2+} 및 인산화 효소의 센서 단백질인 칼슘-의존성 단백질 카이네즈(CDPKs)는 식물 세포에서 가장 풍부한 세린/트레오닌 키나아제이다. 이들은 다양한 자극에 대한 신호를 변환하여 식물에서 특정 반응을 일으킨다. 벼에는 31개의 CDPK 유전자 족이 확인되었다. 그들은 주로 식물의 생장과 발달에 관여하며 다양한 스트레스 조건에 반응하여 기능을 하는 것으로 알려져 있다. 그러나 CDPK 단백질의 생화학적 특성에 대해서는 알려진 바가 별로 없다. 이 연구에서는 벼의 CDPK 중 하나인 OsCPK11을 부분 정제하여 그 생화학적 특성을 조사하고자 하였다. 벼 유식물에서 3단계 칼럼 크로마토그래피 과정을 거쳐 부분 정제된 OsCPK11을 얻었다. 정제 과정에는 DEAE를 사용한 음이온 교환 크로마토그래피, Phenyl-Sepharose를 사용한 소수성 상호작용 크로마토그래피 및 Sephacryl-200HR를 사용한 겔 여과 크로마토그래피를 포함하였다. 부분 정제된 OsCPK11은 분자량이 54kDa이며 소수성 수지와 강한 소수성 상호작용을 보였다. 부분 정제된 OsCPK11으로 *in vitro* kinase assay를 실시한 결과, OsCPK11은 Ca^{2+} -의존성 자가인산화 활성을 가짐을 보여 주었다. OsCPK11은 histone III-S를 인산화 하였으며, 카이네즈 활성의 최적 pH는 7.5-8.0이었다. Native OsCPK11은 이전에 연구된 재조합 OsCPK11과 몇 가지 생화학적 특징을 공유하였는데, 둘 다 Ca^{2+}-의존성 자가인산화 활성을 나타냈다. 또한, 둘 모두 카이네즈 활성을 위한 기질로서 histone III-S를 선호하였으며, Ca^{2+} 의존성을 보여 주었다.